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CONTENTS

PART 1. FEBRUARY, 1958.

PAGE

D. I. ANNEAR: Observations on the preservation by drying of leptospirae and some other bacteria - - - - - - - - - - 1

DENISE C. HINE, MARY C. DAWBARN AND JEAN SMITH: The influence of the composition of the medium on the potency of vitamin B_{12}-active factors in a tube assay with *Escherichia coli* mutant - - - - 5

J. S. MCKENZIE: Some pharmacological properties of pyrrolizidine alkaloids and their relationship to chemical structure - - - - 11

J. R. CASLEY-SMITH: The haematology of the Central Australian aborigine. I. Haemoglobin and erythrocytes - - - - - - - - 23

ALLAN J. DAY AND J. ANDREW PETERS: The effect of uranium acetate on heparin induced lipemia clearing - - - - - - - - 39

J. W. MAY: Mutation to thiamine independence in *Escherichia coli* - - 49

MARGARET BEECH, A. E. DUXBURY AND PETER WARNER: Murray Valley encephalitis antibodies in an aboriginal mission in South Australia. The application of the haemagglutination-inhibition test - - - 55

M. MESSER: A comparison of the effects of barbiturates, salicylates, chlorpromazine, 2,4-dinitrophenol and cyanide on the synthesis of glutamine by brain cells *in vitro* - - - - - - - - - 65

E. MUNCH-PETERSEN AND J. ARMSTRONG: The influence of orally administered oxy-tetracycline on the rumen bacteria of sheep - - - 77

I. R. MCDONALD, J. R. GODING AND R. D. WRIGHT: Transplantation of the adrenal gland of the sheep to provide access to its blood supply - 83

U of M Library JUL 1 59

PART 2. APRIL, 1958.

PAGE

I. G. WHITE AND D. E. GRIFFITHS: Guanidines and phosphagens of semen 97

D. R. LAMOND: Infertility associated with extirpation of the olfactory bulbs in female albino mice - - - - - - - - - 103

COLIN J. SCHWARTZ, J. ANDREW PETERS AND ALLAN J. DAY: The effects of hypercholesterolaemia on arterial ground substance in rabbit atherosclerosis - - - - - - - - - - - - - 109

COLIN J. SCHWARTZ AND J. R. CASLEY-SMITH: Atherosclerosis and the serum mucoprotein levels of the Australian aborigine - - - 117

ALLAN J. DAY AND J. ANDREW PETERS: Observations on clearing factor inhibitor elaborated by cortisone in rabbits - - - - - - 121

WINIFRED G. NAYLER: Further observations on the seasonal variation in drug sensitivity of the isolated toad heart (*Bufo marinus*) - - 133

J. J. LAWRENCE: The infection and growth of ectromelia virus in the chorio-allantoic membrane of the developing hen's egg - - - 145

PATRICIA E. LIND AND F. M. BURNET: Genetic suppression of virulence in the influenza virus strain WS— - - - - - - - - 159

I. KALDOR: Studies on intermediary iron metabolism. XII. Measurement of the iron derived from water soluble and water insoluble non-haem compounds (ferritin and haemosiderin iron) in liver and spleen - 173

T. R. BRADLEY: The effect of insulin on the metabolism of mammary gland slices from hypophysectomized rats - - - - - - 183

PART 3. JUNE, 1958.

PAGE

R. K. F. PAK POY: Electron microscopy of the piscine (*Carassius auratus*) renal glomerulus - - - - - - - - - - - - 191

D. I. ANNEAR: Observations on drying bacteria from the frozen and from the liquid state - - - - - - - - - - - - - 211

JEAN YOUATT: The uptake of isoniazid by washed cell suspensions of *Mycobacteria* and other organisms - - - - - - - - 223

G. J. V. NOSSAL: The induction of immunological tolerance in rats to foreign erythrocytes - - - - - - - - - - - - 235

BARBARA McDOUGALL AND F. GIBSON: The effect of the isomers of chloramphenicol on growth and indole synthesis by *Escherichia coli* 7-4 - - - - - - - - - - - - - 245

A. SHULMAN AND GLENDA M. LAYCOCK: The analeptic activity of bemegride 251

E. L. FRENCH AND J. K. DINEEN: Primary isolation of influenza A virus: the optimal conditions for amniotic infection - - - - - - 257

R. G. WEBSTER: The immunological relations of the contagious pustular dermatitis virus to the mammalian pox group - - - - - - 267

E. R. TRETHEWIE: Cortisone and anaphylaxis - - - - - - - 275

HENRY FRANCIS HOLDEN: Apparatus for vacuum distillation and for "freeze-drying" - - - - - - - - - - - - - - 285

PART 4. AUGUST, 1958.

PAGE

JENNIFER TAPLIN AND N. E. GOLDSWORTHY: A study of 225 strains of *Staphylococcus* isolated from the mouth - - - - - - - 289

E. M. TRAUTNER, PAMELA R. PENNYCUIK, R. J. H. MORRIS, S. GERSHON AND K. H. SHANKLY: The effects of prolonged sub-toxic lithium ingestion on pregnancy in rats - - - - - - - - - - 305

WINIFRED G. NAYLER: The effect of potassium on the response of the isolated heart to strophanthin-G - - - - - - - - - - 323

A. BOLLIGER AND R. GROSS: Ether soluble compounds associated with the plumage of birds: a steroid constituent - - - - - - - 333

D. R. GRANT-FROST AND E. J. UNDERWOOD: Zinc toxicity in the rat and its interrelation with copper - - - - - - - - - - - 339

A. SHULMAN AND GLENDA M. LAYCOCK: Bemegride analepsis to deep hypnosis induced by a variety of hypnotics - - - - - - 347

B. G. FIRKIN: The nature of the adsorbed antibody in acquired haemolytic anaemia - - - - - - - - - - - - - - - 359

F. N. FASTIER AND M. A. McDOWALL: A comparison of the pharmacological properties of the three isomeric aminopyridines - - - - 365

G. T. ARCHER AND OLGA KOOPTZOFF: Blood group antigens in white cells - 373

K. D. G. EDWARDS AND H. M. WHYTE: The measurement of creatinine in plasma and urine - - - - - - - - - - - - - 383

MARGARET C. HOLMES: Type specific antibodies to *Streptococcus pyogenes* 395

PART 5. OCTOBER, 1958.

PAGE

G. S. CHRISTIE: Liver damage in acute heliotrine poisoning. 1. Structural changes - - - - - - - - - - - - - - - - 405

G. S. CHRISTIE: Liver damage in acute heliotrine poisoning. 2. Biochemical changes - - - - - - - - - - - - - - 413

V. A. STRANGIO: A study of the period of action in a series of sex-linked recessive lethals in *Drosophila* - - - - - - - - - 425

I. G. JARRETT AND O. H. FILSELL: Hexokinase activity of sheep, lamb and rat tissue - - - - - - - - - - - - - - - 433

E. R. TRETHEWIE AND W. A. ST. CLAIR: Effect of extracellular potassium on the intracellular action current in the beating heart in the intact toad - - - - - - - - - - - - - - - - 441

P. WOOLLEY AND H. WARING: Responses of the perfused fowl leg to posterior lobe pituitary extracts - - - - - - - - - 447

J. M. BOWNESS, G. K. S. CHING AND J. W. H. LUGG: The similarity of a relationship between body weight and urinary 17-ketosteroid excretion in adult male and female human subjects - - - - - 457

P. C. VINCENT AND C. R. B. BLACKBURN: The effects of heavy metal ions on the human erythrocyte. I. Comparisons of the action of several heavy metals - - - - - - - - - - - - - - - 471

BASIL S. HETZEL, JOHN S. CHARNOCK, HUGH R. GILMORE AND KARIN LE MERCIER: Alteration of metabolic response to adrenaline in man by pretreatment with triiodothyronine and hydrocortisone - - 479

J. D. ABERNETHY AND F. N. FASTIER: Effects of amarin, 4-methyl-2-amino-pyridine ('W-45'), tetraethyl-urea, and bemegride on mice given toxic doses of pentobarbitone sodium - - - - - - - - 487

F. N. FASTIER AND M. A. MCDOWALL: Analgesic activity of 4-methyl-2-amino-pyridine and of some related compounds - - - - - 491

P. FANTL AND H. A. WARD: The thromboplastic component of intact blood platelets is present in masked form - - - - - - - - 499

MERRAN NELSON AND F. A. LEWIS: A relationship between swine and Asian strains of influenza A virus - - - - - - - - - 505

PART 6. DECEMBER, 1958.

PAGE

MARY C. DAWBARN, DENISE C. HINE AND JEAN SMITH: Folic acid activity in the liver of sheep. 1. The measurement of folic acid and citrovorum factor - - - - - - - - - - - - - - 511

MARY C. DAWBARN, DENISE C. HINE AND JEAN SMITH: Folic acid activity in the liver of sheep. 2. The effect of "4-amino"-pteroylglutamic acid *in vitro* - - - - - - - - - - - - - 529

MARY C. DAWBARN, DENISE C. HINE AND JEAN SMITH: Folic acid activity in the liver of sheep. 3. The effect of vitamin B_{12} deficiency on the concentration of folic acid and citrovorum factor - - - - - 541

E. A. NORTH: Adverse effect of filtration on the immunizing power of staphylococcal toxin and toxoid - - - - - - - - - - 547

MAGDA REICH: The quantitative estimation of plasma corticosteroids by a modified chromatographic technique - - - - - - - - 555

WINIFRED G. NAYLER: The action of reserpine on the isolated heart - - 567

HUGH R. GILMORE AND COLIN J. SCHWARTZ: The possible clinical significance of elevations of the serum mucoprotein and hexosamine fractions in human disease - - - - - - - - - - 575

P. FANTL: Thrombin and the thromboplastic activity of plasma - - 581

P. C. VINCENT: The effects of heavy metal ions on the human erythrocyte. 2. The effects of lead and mercury - - - - - - - - - 589

DEIRDRE R. NICHOLAS AND W. R. PITNEY: Microbiological assay of vitamin B_{12} content of serum using *Euglena gracilis* - - - - - - - 603

R. ORTMAN AND W. E. GRIESBACH: The cytology of the pars distalis of the wallaby pituitary - - - - - - - - - - - 609

OBSERVATIONS ON THE PRESERVATION BY DRYING OF LEPTOSPIRAE AND SOME OTHER BACTERIA[1]

by D. I. ANNEAR[2]

(From the Department of Pathology, University of Cambridge).

(*Accepted for publication 20th November, 1957.*)

This paper contains progress reports on the preservation of leptospirae and other bacteria by drying methods already described; it is divided, for convenience, into two parts.

PART I. LEPTOSPIRAE.

In a previous study (Annear, 1956a) it was reported that leptospirae were dried in peptone-starch plugs and recovered from them after a storage period of 12 months. The relationship between the size of the inoculum and the time taken for visible growth to appear suggested that the peptone in which the organisms were dried inhibited growth when it was inoculated along with the organisms into the recovery medium.

The present paper reports, from the same series of experiments, some recoveries from desiccates which were examined after storage for 24 months. In two of the experiments, some of the desiccates were stored at 4° C. and some at room temperature.

It is regretted that when the earlier paper was written, the author was unaware of a report of successful drying of leptospirae some twenty years previously by Hindle (1934) and it is now a pleasure to acknowledge this work. Hindle's investigations, however, did not extend beyond a storage period of 15 days.

Materials and Methods.

The organisms, drying methods and recovery medium were described previously (Annear, 1956a).

For the present studies, three desiccates from each experiment were opened and dissolved with 0·5 ml. of Korthof's medium. Then 1, 2, 3 or 4 drops of the dissolved desiccates were sown into 2 ml. volumes of the same medium contained in 4 in. × ⅝ in. test tubes, 5 tubes being done for each desiccate (2 tubes were each sown with 3 drops). The tubes were fitted with rubber stoppers and incubated at 30° C. Daily examinations were made for visible growth for a period of 60 days. Turbid cultures were examined microscopically.

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[1] This work was carried out during tenure of a John Lucas Walker Studentship.

[2] Present address: Institute of Medical and Veterinary Science, Adelaide.

Results and Discussion.

Recoveries of leptospirae were obtained in all experiments and from 16 out of the 18 desiccates opened (Table 1).

TABLE 1.

Recoveries of leptospirae from desiccates after storage for two years.

Exp.	Organism	Storage temp.	Desiccate "A" drops of inoculum					Desiccate "B" drops of inoculum					Desiccate "C" drops of inoculum				
			1	2	3	3	4	1	2	3	3	4	1	2	3	3	4
1	*L. canicola* (Utrecht)	Room	15	N	14	14	14	N	14	15	N	N	15	15	14	N	N
2	*L. leeds*	Room	N	15	14	14	14	15	10	N	N	14	N	N	N	14	15
3a	*L. leeds*	Room	N	N	10	N	15	N	N	10	N	N	N	10	N	15	10
b		4°C	10	10	10	10	10	N	15	10	10	10	10	15	10	10	10
4a	*L. icterohaemorrhagiae* (Jackson)	Room	N	N	N	N	N	N	N	25	N	24	N	N	N	N	N
b		4°C	10	10	14	15	15	N	N	14	16	N	14	14	16	14	15

Numbers in body of table indicate number of days before visible growth appeared in the cultures.
N = No visible growth within 60 days.

In Experiments 3 and 4 the beneficial effect of low temperature storage may be seen.

There was no consistent relationship between the size of the inoculum and the time lag before visible growth appeared as there usually was with the previous recoveries where, however, fewer observations were made. Indeed, in the present results there were gross differences between the members of 7 of the 18 pairs of tubes which were inoculated with 3 drops of the dissolved desiccates. Factors other than peptone concentration and numbers of organisms appear to be involved in the irregular patterns of recovery.

Although it is of considerable interest that the organisms were recovered from desiccates after storage for 2 years, the recoveries with the possible exception of those from material stored at 4° C. seem scarcely consistent or rapid enough to commend this method, as it stands, for long-term preservation of leptospirae.

PART II. OTHER BACTERIA.

This part of the paper is concerned with further studies on two drying methods which have been described previously (Annear, 1956c; Annear, 1957). A small range of organisms has been dried by each of these methods in parallel and recoveries have been measured both immediately after drying and after storage.

Materials and Methods.

Organisms. These are listed in Table 2.

TABLE 2.

Recoveries of bacteria after drying and storage.

Organism	Method of Drying	Storage (months) 0	1	3	6	9	12
		Recovery (per cent.)					
Staphylococcus aureus	1	—	—	—	96	—	94, 109
	2	—	—	—	96	—	100, 49
Chromobacterium prodigiosum	1	92	—	—	86	—	81, 89
	2	81	—	—	30	—	44, 10
Salmonella edinburg (N.C.T.C. 7407)	1	97	—	82	—	—	90, 99, 99
	2	92	—	75	—	—	66, 45
Shigella flexneri (N.C.T.C. 8192)	1	86	—	84	—	—	88, 90
	2	77	—	88	—	—	69, 70
Vibrio metchnekovi (N.C.T.C. 8443)	1	25	10	9	—	—	2
	2	15	5	2	—	—	0·8
Vibrio cholerae (624)	1	42	36	—	31	—	15
	2	18, 23	28	—	19	—	5
Vibrio cholerae (623)	1	7, 15	—	—	—	3, 6	—
	2	18, 17	—	—	—	4, 1	—
Vibrio cholerae (N.C.T.C. 8367)	1	17, 12	—	—	—	8, 15	—
	2	22, 9	—	—	—	1, 0·2	—

Suspension for drying and viable counts. The organisms were dried in a mixture of Evans peptone and glucose (10 p.c. of each). The numbers of organisms dried were of the order of 10^4-10^5/drop. All counts were made on blood-agar. Other details concerning the preparation of suspensions and methods for viable counts have been described (Annear, 1956b; Annear, 1957).

Drying assembly. The drying assembly was as described previously (Annear, 1956b) except that the manifold was seated directly over the P_2O_5 trap and not connected to it by pressure tubing.

Drying methods. Single drops of bacterial suspension were dried in ampoules of 8 ml. capacity. Although seemingly large for such small volumes, these ampoules were very convenient for quantitative studies. Ampoules for the two methods of drying were inoculated in a random manner and one at a time were connected to the manifold and exposed to high vacuum. The desiccates were sealed after a drying period of 24 hours. The two drying methods are outlined below:—

Method 1. Drying on cotton wool tufts (Annear, 1957).

Method 2. The drops of bacterial suspension were dried on the rounded bottoms of bare ampoules—similar to the method described previously (Annear, 1956c).

Storage of desiccates. The desiccates were stored in darkness and at room temperature (*ca.* 20° C.).

Results and Discussion.

Table 2 shows the recoveries of eight organisms dried by the two methods in parallel. On some occasions more than one ampoule from each set was opened for counting. The recoveries of the first four organisms in the Table were high and consistent for Method 1 but variable for Method 2. With the vibrios there were wide variations among replicate ampoules, among the organisms and between the methods but again the recoveries for Method 1 were considerably higher than those for Method 2. The different results obtained for the two methods may be explicable in terms of the physical nature of the dried material; the thin scales on the glass may be more susceptible to 'decay' than the compact material on the fibres.

In view of the results shown here, the previous recommendation (Annear, 1956c) given to Method 2 as a simple means of preserving bacteria could now be withdrawn in favour of Method 1. Some evidence was obtained, however, which showed that greater homogeneity among replicates resulted when the inoculated cellulose tufts were exposed as a batch to high vacuum (Annear, 1957) rather than one by one as was done here.

The relatively poor recoveries of the vibrios are further examples of their widely observed sensitivity to drying procedures; as Fry (1954) has pointed out, the vibrios are a severe challenge to any drying technique.

GENERAL SUMMARY.

Three strains of leptospirae were recovered from dried peptone plugs after storage for 2 years. The shortest lag period, before visible growth, was 10 days and there were unexplained irregularities with respect to the volumes of the dissolved desiccates which were inoculated into the recovery medium and the period elapsing before visible growth appeared.

Single drops of a number of species of bacteria suspended in a mixture of glucose and peptone were dried *in vacuo* over P_2O_5 (1) on tufts of cotton wool and (2) on the glass surfaces of the bottom of ampoules. Higher and more consistent recoveries were obtained with the former method than with the latter, both immediately after drying and after storage for 12 months.

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THE INFLUENCE OF THE COMPOSITION OF THE MEDIUM ON THE POTENCY OF VITAMIN B_{12}-ACTIVE FACTORS IN A TUBE ASSAY WITH *ESCHERICHIA COLI* MUTANT

by DENISE C. HINE, MARY C. DAWBARN AND JEAN SMITH

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(*Accepted for publication 7th November, 1957.*)

The method of Burkholder (1951) for the assay of vitamin B_{12}-activity by an *Escherichia coli* mutant has been in use in this laboratory for several years. Recently the effect of a modified medium on the toxicity of copper for our organism (mutant No. 8134 from the National Collection of Industrial Bacteria, D.S.I.R., England) has been reported (Hine, Dawbarn and Smith, 1957). The altered procedure involved adding a sterile solution of glucose aseptically to tubes autoclaved with medium containing 5 mg. crystalline sodium acetate per ml. but no glucose. This paper will describe the effect on the response to vitamin B_{12}, and to preparations with vitamin B_{12}-activity, of this medium and of one without acetate to which the glucose was added after autoclaving.

METHODS.

The assay procedure has been described in detail (Hine, Dawbarn and Smith, 1955). When glucose was to be added after autoclaving, a sterile 10 p.c. solution was prepared and the medium made up to 4/5 its normal volume, so that the tubes at the time of autoclaving contained 4·5 ml. The glucose was added subsequently in a volume of 0·5 ml.

Aqueous extracts of liver and of rumen contents were prepared by the method already described (Hine and Dawbarn, 1954). The use of the technique of paper ionophoresis has also been reported (Dawbarn, Hine and Smith, 1957). Factor B was prepared as follows: 2 ml. concentrated HCl were added to 2·5 mg. crystalline vitamin B_{12}. After standing for 17 hours at room temperature the solution was diluted with water, 0·5 ml. 1 p.c. KCN was added, the pH was adjusted to 6·5 and the volume made to 500 ml. This solution was completely devoid of activity for *L. leichmannii*, indicating that all the vitamin B_{12} had been destroyed. It promoted the growth of *E. coli* in a plate and a tube assay and was therefore assumed to contain Factor B, denucleotided vitamin B_{12}. No attempt was made to separate this from the other degradation products of the vitamin necessarily present in the solution (Armitage, Cannon, Johnson, Parker, Lester Smith, Stafford and Todd, 1953).

Three media were employed—"standard" medium, with glucose autoclaved in the tubes and no acetate; "separate glucose" medium, with glucose added after autoclaving and without acetate; and "modified" medium, containing 5 mg. sodium acetate per ml. and with glucose added after autoclaving. These media all contained thioglycollic acid. All estimates of the vitamin B_{12} activity of the various preparations tested were calculated from standard lines obtained with the appropriate media.

RESULTS.

The effect of modification of the medium on the response to vitamin B_{12}. Fig. 1 illustrates the change in the dose response line. When glucose was added separately the concavity present in the standard line was eliminated. The addition of acetate resulted in a greatly increased response to vitamin B_{12}. These three lines were always of the characteristic shapes seen in Fig. 1.

With modified medium the maximum growth obtained was always much higher than with the other two media. The positions of the lines for the standard and separate glucose media varied from assay to assay, and either one might give the higher maximum response.

The effect on the estimation of vitamin B_{12}-activity in liver. It will be seen from Table 1 that the estimate of vitamin B_{12}-activity in liver was unaffected by the modification of the assay medium.

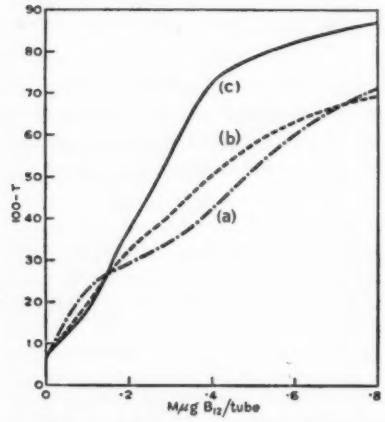

Fig. 1. Growth response of *E. coli* mutant to vitamin B_{12} in (a) standard medium, (b) separate glucose medium, (c) modified medium.

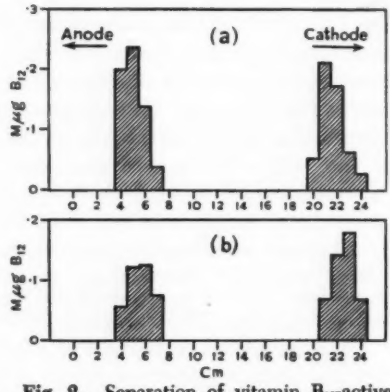

Fig. 2. Separation of vitamin B_{12}-active factors from rumen contents of sheep by ionophoresis on paper: *E. coli* tube assay.

(a) standard medium

$$\frac{\text{activity} -4 \text{ to} -7 \text{ cm.}}{\text{activity} -20 \text{ to} -24 \text{ cm.}} = 1{\cdot}17.$$

(b) modified medium

$$\frac{\text{activity} -4 \text{ to} -7 \text{ cm.}}{\text{activity} -20 \text{ to} -24 \text{ cm.}} = 0{\cdot}82.$$

Sheep fed on wheaten hay chaff and gluten with 1 mg. Co *per os* daily.

The effect on the estimation of vitamin B_{12}-activity in rumen contents. The estimated activity in rumen contents from a sheep on pasture was 4 p.c. higher with the separate glucose medium and 13 p.c. higher with modified medium than with standard medium (Table 1). For rumen contents from an animal fed a

TABLE 1.

Estimates of B_{12}-activity with different media.

Medium	Assay No.	Liver	Rumen contents from Co treated sheep	Rumen contents from sheep on pasture	Factor B
		mμ vitamin B_{12}-activity/ml. extract			
Standard	1	61·1	33·1	37·2	411
	2	57·5	33·1	36·3	416
	3	55·5	37·5	34·1	430
	4	56·9	35·7	36·4	435
Separate glucose	2	59·7	43·0	37·7	592
	4	55·5	45·7	37·7	611
Modified	1	57·2	48·4	40·3	684
	3	56·3	48·8	40·7	669
Means:					
Standard		57·75	34·85	36·0	423
Separate glucose		57·6	44·35	37·7	601·5
Modified		56·75	48·6	40·5	676·5
(Non-significant differences are indicated by brackets.)					
Coefficients of Variation:					
Combined data		4·0%	4·6%	2·8%	2·2%

cobalt-deficient diet of wheaten hay chaff and gluten and given cobalt supplements by mouth, the corresponding increases were 29 p.c. and 38 p.c.

When this extract was subjected to ionophoresis on paper, it was found that with standard medium the spot representing cyanocobalamin (—4 to —7 cm.) was larger than that comprising residual activity, but with modified medium this relationship was reversed (Fig. 2). Different amounts were applied to the paper for the two media, so that only the distribution of the activity should be compared.

The effect on the estimation of the vitamin B_{12}-activity of a Factor B preparation. An increase of 40 p.c. in the estimated activity of the Factor B preparation resulted from adding glucose separately, and modified medium gave a 60 p.c. increase (Tables 1 and 2). The slope of the response line for the Factor B preparation relative to that for vitamin B_{12} was also changed (Table

TABLE 2.

Estimated activity of a factor B preparation.

Dilution of Factor B preparation	Standard medium	Separate glucose medium	Modified medium
	mγ vitamin B_{12}-activity/ml.		
1 in 12,500	300	580	600
2 in 12,500	373	513	800
3 in 12,500	370	537	678
4 in 12,500	363	534	594
5 in 12,500	400	570	560
6 in 12,500	390	585	517
7 in 12,500	461		
8 in 12,500	461		
MEAN	390	553	625

2). With standard medium increasing concentration resulted in increasing estimates. When glucose was added separately without acetate the estimates at different concentrations showed less variation. With modified medium decreasing values were obtained with increasing concentration.

DISCUSSION.

Ford (1953) reported that the vitamin B_{12} activities of Factor A and pseudovitamin B_{12} for *L. leichmannii* may depend on the composition of the growth medium. The modifications of Burkholder's medium described here have had a similar effect with a mutant of *E. coli*. For a tissue which contains predominantly true vitamin B_{12}, e.g. liver, the estimate of vitamin B_{12}-activity obtained was independent of the medium used, but with extracts of rumen contents the estimate was increased by adding glucose separately after autoclaving, and further increased if acetate was incorporated in the medium. Modification of the medium had less effect on the estimate of vitamin B_{12}-activity in the rumen contents of a sheep on pasture than was the case with one fed on wheaten hay chaff with added cobalt. This finding is in accord with earlier observations that the rumen contents of pasture fed animals contain a much higher proportion of true vitamin B_{12} (Dawbarn, Hine and Smith, 1957). Paper ionophoresis of one extract showed that with modified medium a greater part of the activity was due to factors other than true vitamin B_{12} than was the case with standard medium.

The vitamin B_{12}-activity of a solution containing Factor B was increased by the modification of the medium. Factor B seems to be only a minor component of the activity in rumen contents (Dawbarn *et al.*, 1957), and indeed paper ionophoresis of the sample examined in these tests failed to disclose a

separate fraction attributable to Factor B, although it may possibly form a small part of the activity concentrated at — 20 to — 24 cm. The effect of the modified medium on the estimation of vitamin B_{12}-activity in rumen contents must therefore be attributed mainly to the other active factors present.

SUMMARY.

Two modifications of the standard medium were employed: "separate glucose" medium which involved adding the glucose aseptically to the tubes after autoclaving; and "modified" medium, which contained sodium acetate and had glucose added after autoclaving. Each medium had a characteristic effect on the shape of the dose response line to vitamin B_{12} with *E. coli* mutant.

The estimate of vitamin B_{12}-activity in liver was the same for all three media. However, with extracts of rumen contents and a Factor B preparation, the separate glucose and modified media gave increased estimates, modified medium giving higher values than separate glucose medium. In the case of rumen contents from a sheep fed wheaten hay chaff and given cobalt by mouth, paper ionophoresis showed that with modified medium a greater proportion of the activity was due to factors other than true vitamin B_{12} than with standard medium.

Acknowledgment. We wish to thank Mr. G. N. Wilkinson, of the Division of Mathematical Statistics, C.S.I.R.O., for the statistical analysis.

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SOME PHARMACOLOGICAL PROPERTIES OF PYRROLIZIDINE ALKALOIDS AND THEIR RELATIONSHIP TO CHEMICAL STRUCTURE

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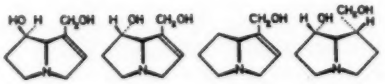
Pyrrolizidine alkaloids are known or suspected to be the toxic principles in many plants which cause liver disease in grazing stock, in Australia and other parts of the world (Hopkirk and Cunningham, 1936; Shtenberg and Shillinger, 1950; Gromov and Shakhnazarova, 1951; Chen, 1945; Sapeika, 1952; Bull, 1954; Bull *et al.*, 1956; Schoental, 1957).

Interest has been largely concentrated on their hepatotoxic properties and what little information is available concerning other pharmacological activities, principally relating to the Senecio alkaloids, indicates inhibitory action on intestinal smooth muscle, weak atropine-like properties, temporary depressor activity and weak stimulation of guinea-pig uterus (*Anon.*, 1949). The alkaloid platyphylline has received more attention than any other of the group. It has relatively strong atropine-like activity, and has found therapeutic use in the U.S.S.R. as an antispasmodic and mydriatic (Anichkov and Belenky, 1955; Petrovsky, 1956).

The pyrrolizidine alkaloids are esters of amino-alcohols such as heliotridine (I), retronecine (II), supinidine (III) and platynecine (IV) with carboxylic acids of some complexity.



I II III IV

The N-oxides of the alkaloids frequently occur in nature along with the parent bases (Areshkina, 1951; Culvenor, 1954). This linkage of an oxygen atom to the tertiary nitrogen diminishes the basic properties of the molecule (Culvenor, 1953).

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We have studied the acute and chronic effects on rats of the alkaloids of *Heliotropium europaeum* L. as part of an investigation of the disease in sheep grazing on pastures in which the plant occurs. These studies are being extended to other members of the group.

In the present paper, with the rat's ileum, the smooth-muscle inhibitory and anticholinergic activities of several pyrrolizidine alkaloids are compared. The substances examined include two new alkaloids, heleurine and europine (Culvenor, 1954; Crowley and Culvenor, 1956), and three N-oxides.

Materials and Methods

Rat's Ileum Preparation.

Rats of either sex (but usually males) were killed by a blow on the nape of the neck, were bled from the jugular veins, and a length of about 10 cm. was removed from the upper ileum. From this about 2 cm. was selected, freed from excess mesentery, suspended in a 10 ml. bath of Tyrode solution (Gaddum, 1954), at 37·5 ± 0·5° C., and connected to a frontal writing lever. The Tyrode was aerated by oxygen.

All compounds tested were added as aqueous, usually neutral, solutions. No volume greater than 0·5 ml. was added to the bath at any one time.

Assessment of Inhibitory and Anticholinergic Activities.

Inhibitory action on intestinal musculature consequent to addition of a substance was measured as the maximum fall in the tracing within one minute of its addition. At the end of that minute the bath was washed 3 times with fresh Tyrode.

Antagonism to acetyl choline was measured as the percentage reduction of the response to a standard dose of acetyl choline. Because the antagonistic effect of most pyrrolizidine alkaloids persists somewhat after wash-out, each test of antagonism was preceded and followed by a response to the acetyl choline dose alone. The response in presence of antagonist was compared with the mean of these two standard responses. Since pyrrolizidine alkaloids at anticholinergic dose-levels cause some fall in tonus, the response to acetyl choline was measured from the base-line of diminished tonus.

Antagonists were added one minute before acetyl choline. In all instances intervals of 3 or 4 minutes occurred between tests, whether of inhibition, of antagonism to acetyl choline, or of response to the standard dose of acetyl choline. Rat's ileum was found remarkably insensitive to acetyl choline; a sub-maximal dose of 20 μg was used. This was left in the bath until the response reached a maximum or for one minute, whichever was shorter.

In most comparisons of potency, whether for inhibition or for antagonism to acetyl choline, lasiocarpine was used as a "standard", but in some experiments heliotrine was so used. These two alkaloids were compared with each other in a number of experiments; also with adrenaline for inhibition of tonus, and with atropine for antagonism to acetyl choline.

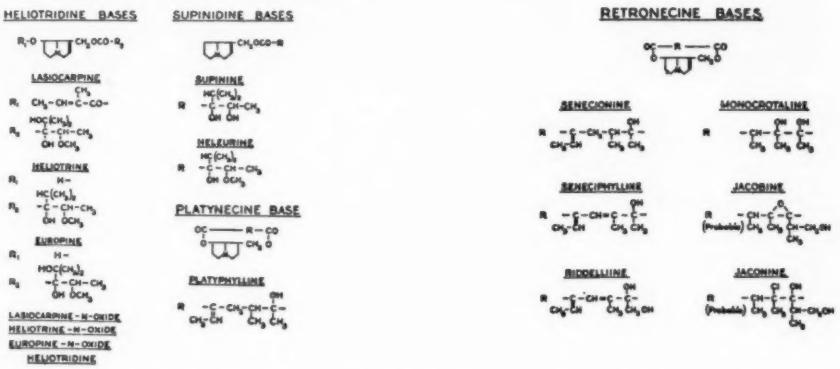
Treatment of Results.

Comparisons of potency in any one experiment were obtained from log dose-response relationships over an active range of doses for each alkaloid. In some cases of anticholinergic action these relationships did not differ significantly from linearity or parallelism, and were susceptible to conventional bio-assay procedures. Where deviations from linearity or parallelism made this impossible, the results were represented graphically. An approximate estimate of relative anticholinergic potency was then made by comparison of doses required to produce 50 p.c. inhibition of the response to the standard dose of acetyl choline. In some experiments where each substance was examined at only 2 dose-levels, linearity was assumed, and the results tested for parallelism only.

In the experiments on inhibition of tonus, none of the results was suitable for the usual statistical treatment. The figures presented for relative potency are approximate estimates obtained from graphical representation of the log dose-response relationships.

Pyrrolizidine Alkaloids and Derivatives Used.

The following pyrrolizidine alkaloids, based on heliotridine (I), retronecine (II), supinidine (III), or platynecine (IV), were used:



Heliotric acid, the esterifying acid of heliotrine, was also tested in neutral aqueous solution.

RESULTS.

Inhibition of Intestinal Muscle Tonus.

All the pyrrolizidine alkaloids tested inhibited the rat's ileum. The inhibition of average tonus was sometimes accompanied by inhibition of pendular movements if present, but often the tonus fell with no change in rhythm or amplitude of beat. The usual order of dose to produce near-maximal inhibition was 1 to 5 mg.

Since the alkaloids were used in aqueous solution it was possible that the effects, especially at high doses, could be due to dilution of the Tyrode. Experiments eliminated this possibility; the only dilution-effect observed on adding up to 1 ml. of distilled water to the 10 ml. bath was a slight increase in tonus.

On wash-out, the inhibitory effect tended to persist for several minutes, with gradual recovery of tonus (Fig. 1). This after-effect was more noticeable with the more potent alkaloids such as senecionine. The inhibition, particularly at high doses, and the persistence of the after-effect varied between preparations. With some, particularly monocrotaline, wash-out was followed by a further decrease in tonus before recovery.

Adrenaline added to the bath in doses of about 5 μg. caused a qualitatively similar inhibition (Fig. 2). Tonus usually recovered more rapidly after wash-out of adrenaline than after wash-out of equi-active doses of pyrrolizidine alkaloids.

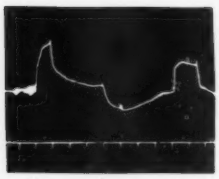


Fig. 1. Inhibition of longitudinal tonus in isolated ileum of rat produced by senecionine. The slow recovery of tonus after wash-out, and reduced response to acetyl choline, are evident. In this and subsequent Figures, Tyrode was changed three times at the points indicated by wash-out artifacts. Time marker indicates periods of one minute.

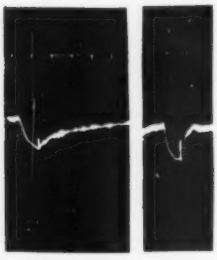


Fig. 2. Longitudinal tonus of isolated rat's ileum is inhibited to approximately the same extent by 5 mg. of lasiocarpine and 5 µg. adrenaline; tonus recovers almost at once after wash-out of adrenaline, but slowly after lasiocarpine. Time marked at intervals of one minute.

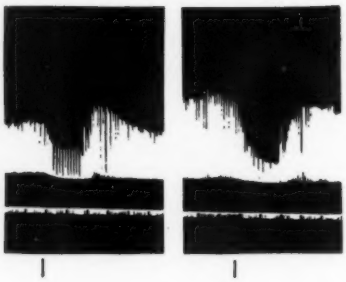


Fig. 3. Lasiocarpine reduces amplitude and slows rate of beat in isolated rabbit ileum. At a dose which more severely reduces amplitue of beat, adrenaline has no effect on its rate. Large interval in time–marker represents one minute.

Antagonism to Acetyl Choline.

In doses causing maximal inhibition of tonus, the alkaloids blocked completely the contractile response to 20 µg. of acetyl choline. In doses causing little or no inhibition of tonus, the alkaloids reduced this response. The spasmodic effect of acetyl choline was reversed with overshoot when relaxing doses of heliotrine were added after 1 minute. The anticholinergic effect partly persisted after wash-out, particularly with the more potent alkaloids such as supinine. Lasiocarpine usually caused 50 p.c. reduction in the acetyl choline response at doses of about 250 µg., but occasionally doses as low as 100 µg. or as high as 1 mg. were necessary.

Estimates of Potency.

Inhibitory Potency. Potencies were compared in terms of the doses required to produce a 5 mm. fall in the tracing. The results of these comparisons are shown in Table 1. All potencies are expressed with respect to that of lasiocarpine. Where an alkaloid was not compared directly with lasiocarpine, but with heliotrine or adrenaline, the potency relative to that of lasiocarpine was obtained by reference to the mean of the estimations for heliotrine or adrenaline. These instances are shown in Table 1. The relative potencies were calculated on a weight basis. Expression on a molarity basis had no effect on the ranking of the potencies, and little effect on their magnitude (Table 1).

TABLE 1.

Tonus-inhibiting and anticholinergic potencies of pyrrolizidine alkaloids and derivatives.

Substance	pK†	Inhibitory action		Anticholinergic action		
		Relative potency wt. basis	Relative potency corrected for M. Wt.	Relative potency (Fid. limits) wt. basis	Relative potency corrected for M. Wt.	Ratio of graphical to statist. potency estimates
Heliotridine Bases						
Lasiocarpine	7·5	1·00	1·00	1·00	1·00	
Heliotrine	8·2	0·4		2·85(1·76-5·01)	2·16	1·2
		2·5		2·84(1·99-4·26)	2·16	1·1
		1·0		10·2(2·41-5·08)	7·8	3·9
		0·6		1·43(0·83-2·57)	1·09	0·98
		1·0		5	3·8	
		1·0		16	12	
		(Mean 1)	1	(Median 4)	(Median 3)	
Europine		0·05	0·04	0·165(0·102-0·308)	0·13	1·2
Lasiocarpine-N-oxide		0·006*	0·006	0·001	0·001	
Heliotrine-N-oxide		0·006*	0·005	0·007	0·006	
Europine-N-oxide		0·006	0·005	0·001	0·0008	
Heliotridine	9·0	0		0·01	0·004	
Retronecine Bases						
Senecionine	6·8	50**	41	4**	3·4	
Seneciphylline	7·0	4		4·31 (1·66-20·4)	3·5	0·93
		1				
		(Mean 2·5)	2			
Riddelliine	7·2	0·2	0·2	1	0·9	
Monocrotaline	7·4	3**		0·0927 (0·000-0·377)	0·073	2·2
		1				
		(Mean 2)	2			
Jacobine		0·5		0·352 (0·111-1·23)	0·30	0·57
		0·5				
		(Mean 0·5)	0·4			
Jaconine		1		1·6 (0·88-2·5)	1·6	1·0
		1				
		(Mean 1)	1			
Supinidine Bases						
Supinine	9·2	1		106(38·9-593)	75	1·9
		1		120	80	
		(Mean 1)	1	(Mean 113)	(Mean 78)	
Heleurine	9·4	3		116(57·0-312)	84	1·5
		1		350‡	250	
		(Mean 2)	2	(Mean 230)	(Mean 170)	
Platyphylline		6	5	1,200	1,000	
Heliotric Acid		0		0·0003		
Adrenaline		3,000				
		3,000**				
		1,000**				
		(Mean 2,300)	920			
Atropine	9·6			7,460 (4,520-14,200)	5,200	1·3

† Figures supplied by C. C. J. Culvenor from unpublished data.
* Comparison made with adrenaline.
** Comparison made with heliotrine.
‡ Comparison made with jacobine.

Anticholinergic Potency. The potencies relative to lasiocarpine and their fiducial limits are shown in Table 1. Estimates of relative potency were made by the graphical method in all instances, and by statistical analysis where applicable. When both methods were used, the ratios of the two estimates are shown in the Table. The mean of these ratios did not differ significantly from 1. The potencies obtained by the graphical method only were therefore accepted equally with those obtained by analysis; the broad fiducial range of the latter lends support to this procedure. No fiducial limits could be estimated in the graphical method.

Potencies were calculated on a weight basis, but are also shown expressed on a molarity basis in Table 1.

Effect on Rabbit's Ileum.

Lasiocarpine and heliotrine inhibited the rhythmic pendular movements of rabbit's ileum at bath concentrations of 2×10^{-4}. The size of the contractions was diminished, sometimes with a very slight fall of base-line tonus, and the frequency of beat was slowed by 30-40 p.c. The effects were immediately reversible on wash-out. Adrenaline had a similar effect on amplitude, though at much lower dose-levels, without affecting the frequency of contraction (Fig. 3).

DISCUSSION.

Several authors have reported that pyrrolizidine alkaloids inhibit isolated intestine. Among the Senecio and Crotalaria alkaloids, retrorsine (Chen *et al.*, 1935), seneciphylline (Chen *et al.*, 1940a), senecionine, jacobine, integerrimine, spartioidine (Harris *et al.*, 1942a), monocrotaline (Harris *et al.*, 1942b), and riddelliine (Harris *et al.*, 1943) have all been shown to inhibit isolated rabbit's intestine, and for most of them depressor activity in anaesthetised cats and stimulant action on guinea-pig uterus were observed. The same workers observed a temporary depressor action of lasiocarpine (Chen *et al.*, 1940b). In a review of this work (*Anon.*, 1949) it was added that the pyrrolizidine alkaloids showed some atropine-like activity, already observed with platyphylline (Chen *et al.*, 1940a).

Platyphylline, an ester of platynecine (IV), has been reported to have no hepatotoxic action (Chen *et al.*, 1940a; Grebennik and Zakharova, 1954). The anticholinergic activity of platyphylline is about 1/5-1/20 that of atropine (Kovyrev, 1944; Goldenherschel, 1943; Syrneva, 1946), and its mode of action similar (Babsky, 1940). Its action against barium-induced spasm in rabbit's intestine is greater than that of atropine (Syrneva, 1946). It apparently depresses the vasomotor centre, producing a fall of blood pressure, and in large doses inhibits the heart (Syrneva, 1946).

By analogy with platyphylline it is suggested that the temporary fall in blood pressure of anaesthetised cats which has been reported for most pyrrolizidine alkaloids, and which we have observed with lasiocarpine in anaesthetised

rabbits, is due to depression of the medullary vasoconstrictor centres. A hypotensive action with large doses of atropine is attributed to medullary vasomotor depression (Goodman and Gilman, 1955).

The mode of action of pyrrolizidine alkaloids on isolated ileum cannot be deduced from the present experiments. However, it seems certain that the antagonism to acetyl choline is an action independent of the inhibitory effect on longitudinal muscle tonus. This is shown by the complete lack of correlation between the relative potencies of the alkaloids in the two activities (Table 1), and suggested by the fact that considerable antagonism to acetyl choline could be evinced by doses at which there was little or no relaxation of the gut.

The inhibitory action on tonus, superficially resembling that of adrenaline, differs in the greater persistence of the after-effect, and in the slowing of frequency of beat in rabbit intestine, an effect absent with equi-active doses of adrenaline (Fig. 3). Adrenaline (Gaddum, 1944) and ephedrine (Goodman and Gilman, 1955) inhibit the isolated guinea-pig's uterus. The stimulation of this preparation by pyrrolizidine alkaloids, reported by Chen and co-workers, offers further evidence that the mode of action of the pyrrolizidine alkaloids on smooth muscle differs from that of sympathomimetic amines.

Trautner and McCallum (1950) have described the effects of lasiocarpine and heliotrine on the electrical behaviour of amphibian striped muscle, and compared them with those of atropine. Heliotrine depressed the action potential of toad's sartorius muscle steadily over a period of hours; lasiocarpine and atropine, in addition to the same depressive action, caused a prolongation of the after-potentials, rapid abolition of the electrical response on repetitive stimulation at low frequencies, and prolongation of conduction time. It is difficult to see any reflection of these actions, particularly the different actions of heliotrine and lasiocarpine, in the effects on smooth muscle.

The results presented here on the comparison of the inhibitory and anticholinergic potencies on smooth muscle of a number of pyrrolizidine alkaloids, permit some conclusions to be drawn regarding the relationships between chemical structure and pharmacological action. The accuracy of the comparisons has been reduced by the variability in responsiveness of rat's ileum to the alkaloids. Nevertheless, it is evident from Table 1 that the alkaloids can at least be grouped according to order of potency.

Inhibitory Potency. All the pyrrolizidine alkaloids are less potent than adrenaline in causing relaxation of intestinal smooth muscle. Since the two fragments available, heliotridine and heliotric acid, are inactive, it is inferred that the inhibitory action requires intact ester molecules. The virtual inactivity of the three N-oxides indicates the necessity of an exposed tertiary N atom.

Of the alkaloids, senecionine is distinctly more active than the others. Introduction of a double bond into the main carbon chain of its esterifying acid, as in seneciphylline, reduces activity about 20 times. If now a hydroxyl group is introduced on the α-C-methyl group, as in riddelliine, activity falls another

10 times. Reduction of the ring double bond of senecionine, as in platyphylline, reduces inhibitory potency 8-fold.

The loss of angelic acid, the unsaturated esterifying acid, from lasiocarpine, giving europine, reduces inhibitory potency 20 times. Reduction of the hydroxyl group on the dimethyl-substituted carbon atom of the lasiocarpic acid portion of europine, giving heliotrine, restores activity to that of lasiocarpine. Removal of the hydroxyl group from position 7 of the pyrrolizidine ring of heliotrine to give the supinidine base heleurine has no effect on activity, nor does replacement of the methoxyl group of heleurine by hydroxyl to give supinine.

Monocrotaline, jacobine and jaconine, which differ considerably from seneciphylline in the acid skeleton (Bradbury and Willis, 1956; Bradbury, 1956), do not differ appreciably from it in activity. The breaking of the epoxy ring with addition of a Cl atom in the transition from jacobine to jaconine has little effect, if any.

Antagonism to Acetyl Choline. The alkaloids fall into fairly clear groups also according to their anticholinergic potencies. The insignificant potency of the heliotrine fragments heliotridine and heliotric acid shows again the importance of the intact ester molecule. The marked loss of activity on forming the N-oxides of heliotrine, lasiocarpine and europine, respectively, indicates the importance of a tertiary N atom and suggests the importance of a relatively strong basic centre in the molecule for anticholinergic action. Although all the pyrrolizidine alkaloids tested here are weaker than atropine, the much greater activity of platyphylline than that of senecionine in which the esterifying acid is identical (Orekhov, 1955), shows that saturation of the pyrrolizidine ring (a matter of one bond) increases anticholinergic activity 300-fold.

Comparison of heleurine and heliotrine shows that substitution of hydroxyl on position 7 of 1-methyl pyrrolizidine reduces activity about 40-fold. Replacement of methoxyl by hydroxyl (i.e. heliotric by trachelanthic acid) in supinidine esters apparently reduces potency, although the difference observed between heleurine and supinine may not be reliable.

Unlike its effect on inhibitory activity, reduction of the ring-double bond in the seneciphyllic acid moiety of seneciphylline to give senecionine has no effect on atropine-like activity. Substitution of a hydroxyl group into the α-C-methyl of seneciphyllic acid as in riddlelliine reduces potency about 4 times. Breaking the epoxide group in jacobine to form jaconine increases activity about 5 times.

Esterification of angelic acid with europine to form lasiocarpine increases activity 10-fold; replacement of lasiocarpic acid in europine by heliotric acid as in heliotrine, i.e. reduction of one hydroxyl group in the acid moiety, increases activity about 40 times.

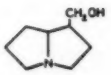
In monocrotaline the proportion of hydroxyl to alkyl substituents on the esterifying acid chain is greatest, and the anticholinergic action is least, of the bases.

In general, hydroxyl substituents appear to decrease activity, with substi-

tution on the pyrrolizidine ring usually exerting a greater relative influence than substitution in the acid moiety. It would be of great interest to compare the anticholinergic activity of platyphylline with that of rosmarinine, which differs in that a hydroxyl group is substituted in position 2 of platynecine (Orekhov, 1955).

There is a general tendency for the more active alkaloids to have the highest pK values (Table 1). The exceptions to this tendency, as in the comparison of senecionine with heliotrine, lasiocarpine, riddelliine and particularly monocrotaline, cast doubt on the importance of pK in determining potency. That the ester grouping is at least as important as basicity is shown by heliotridine, which has a high pK but very low activity.

Macrotomine, an ester of trachelanthamidine (V), bears the same structural relationship to trachelanthamine as europine does to heliotrine, i.e. it has a hydroxyl substituent on the dimethyl-substituted C atom of the esterifying acid. It is therefore an interesting confirmation of the effect of this substitution that macrotomine is less actively anticholinergic than trachelanthamine (Sharapov, 1953).



V

It is most unlikely that the hepatotoxic properties of Heliotrope, Senecio or Crotalaria alkaloids arise out of their atropine-like activity or are related to their ability to inhibit intestinal smooth muscle. Platyphylline, the strongest antagonist of acetyl choline in the series, is the only member so far found not to be hepatotoxic. The intravenous LD50's to mice, which reflect ability to cause hepatic necrosis (*Anon.*, 1949), do not correlate with relative activities observed in this investigation. The only correlation of this type is that the LD50's in rats of the N-oxides of lasiocarpine and heliotrine are greater than those of their respective parent alkaloids. Even so, the LD50 of lasiocarpine-N-oxide in male rats is less than twice that of heliotrine, and in female rats its LD50 is less than that of heliotrine. Further, lasiocarpine is several times more toxic to rats than heliotrine, although it is less actively anticholinergic (Bull *et al.*, 1957).

If the supinidine alkaloids were shown to be hepatotoxic it would strengthen the hypothesis that the 1,2 double bond is necessary for liver damage by these alkaloids. If they are not hepatotoxic, further investigation of the supinidine alkaloids as possible atropine substitutes would be justified. In this latter context, macrotomine, trachelanthamine or any other alkaloids which similarly combine those features of platyphylline and the supinidine esters that appear to enhance anticholinergic activity might be expected to have even more promising activities. This expectation is apparently not satisfied with trachelanthamine

and macrotomine which are less active than supinine (Orekhov, 1950). This may, of course, mean that the steric differences between *l*-heliotridane (from which are derived platyphylline and supinine) and *l*-pseudoheliotridane (base of origin for trachelanthamidine) are of great importance for anticholinergic activity.

Such questions must await the availability of alkaloids with the appropriate structures for their resolution.

SUMMARY.

A number of pyrrolizidine alkaloids, and N-oxides of three of them, have been compared for ability to inhibit longitudinal tonus and to antagonise acetyl choline in rat's ileum. The N-oxides are almost devoid of inhibitory and anticholinergic activities compared with their parent bases. The intact ester-molecule is shown to be necessary for both activities.

The alkaloids are all weaker antagonists of acetyl choline than is atropine, but among them exist considerable differences in potency. Platyphylline, heleurine and supinine are the most potent antagonists of those studied here, and senecionine the most powerful inhibitor of longitudinal tonus. The two activities appear to be independent.

From the present results and from data in the literature some general conclusions are drawn regarding the relationships between chemical structure and pharmacological action in the series. Saturation of the pyrrolizidine ring increases anticholinergic activity markedly. Absence of the ring-substituted hydroxyl group also increases anticholinergic activity greatly, but has no effect on inhibitory action. In general, substitution of hydroxyl groups in the molecules appears to reduce both types of activity, but more severely the antagonism to acetyl choline.

It is not considered that the smooth muscle-inhibiting and anticholinergic actions of the pyrrolizidine alkaloids are directly connected with their hepatotoxic properties.

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THE HAEMATOLOGY OF THE CENTRAL AUSTRALIAN ABORIGINE[1]

I. HAEMOGLOBIN AND ERYTHROCYTES

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There have been a number of haematological studies on different ethnic groups living under various conditions. These have been summarized by Kennedy (1939), The Advisory Committee on Nutrition Surveys of the Nutrition Society (1945), Wintrobe (1956) and others. The most complete account of the effects of varying conditions and diets in a non-European population is to be found in the work of Napier, Das Gupta, Sankaran and others reported in *The Indian Journal of Medical Research* from about 1935 onwards.

So far as can be discovered, there have been only three such studies on the Australian aborigine. These comprise a series of 25 males by Wardlaw (1935), an unpublished series of 34 males and 35 females by Walsh (1957, personal communication), and a recent survey of 134 natives by Davis and Pitney (1957). Consequently, it was felt that advantage should be taken of an opportunity to examine this feature in Central Australian aborigines whose blood was being collected for grouping, antibody and serum lipid studies.

Material and Methods.

The Adelaide University's expedition to Haast's Bluff in 1956 collected data on 145 aborigines. These were mostly western desert natives living in a semi-detribalized state around the Government Settlement which is some 160 miles west of Alice Springs. The main tribal groups included Pintubi, Ngalia, Aranda, Pitjandjara, Luritja and many inter-tribal mixtures. Most had been in that region for about 15 years.

The sexes were about equally divided and ages were estimated from general appearance, personal memory, Settlement records and dental status. Over the age of 25 years, however, estimates were more difficult and are probably not more reliable than $\pm$ 5 years. Blood was obtained by venepuncture. A tourniquet was often necessary, but to obviate any significant alteration in blood constituents, it was not left on longer than half a minute (M.R.C., 1943).

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[1] The data upon which this work is based were collected while upon a University of Adelaide Anthropological Expedition. This was financed by grants from the Wenner-Gren Foundation for Anthropological Research, New York, and the Medical Research Fund of the University of Adelaide.

The European control series was obtained mainly from donors at the Adelaide Red Cross Blood Centre. These were all healthy individuals, none of whom was on iron therapy. Half of them had given blood before but this was at least three to six months previously and had been only 600 ml. (M.R.C., *loc. cit.*, and Walsh *et al.*, 1953, found no significant difference between donors and the general populace). Since there were few female donors between 15 and 25 years of age this group was supplemented by women technicians and students. The control series duplicated the aborigines in age and sex as closely as possible but contained fewer individuals at the extremes of life.

Haemoglobin was estimated by the Sahli method, using one instrument throughout. This was standardized against a photo-electric colorimeter, measuring oxyhaemoglobin formed with dilute ammonium hydroxide. The colorimeter was in constant use and was checked every few weeks against others and against standard bloods supplied by the National Standards Laboratory, Sydney. Those standards are obtained by iron content estimation (see Walsh *et al.*, 1953). On 89 comparisons the Sahli instrument gave a mean of 0·2236 gm./100 ml. low, with a standard error of 0·0112, making the 95 p.c. limits of the mean error to be ± 0·0223 gm.

Cell counts could not be done immediately because of conditions in the field, and the rate at which the aborigines came up for examination. Therefore, the bloods, after dilution, were put into small, clean, dry screw-top bottles with rubber seals to prevent evaporation. They were counted on the night of collection, some in duplicate by a standardized instrument. The control series was treated in identical fashion so that the relative values are comparable but the absolute values may, perhaps, be affected by adsorption of cells on to the glass.

Erythrocyte diameters were measured by projecting the blood films, stained by Leishman's method, on to photographic printing paper. A ×60 dry objective was used, giving an ultimate magnification of × 1688·4, with a standard error of 0·48 (based upon 7 observations spaced throughout the period of photography). The stage micrometer revealed no distortion at the edges of the small field used. In each film the maximum and minimum diameters of 50 cells were measured to the nearest millimetre on the photographs, i.e. to 0·6 μ on the films. In each case the field was selected to secure a minimum of cellular overlapping, and to avoid cells obviously distorted by drying. Starting from one edge of the photograph, the first 50 cells with clearly defined edges were measured. No cell was excluded and thus it was possible to make an estimate of the anisocytosis, eccentricity and "non-circularity" of the cells in the stained dry film. (The term "non-circularity" is used here to denote the amount of "out of roundness" of the cells, whether due to poikilocytosis or ovalocytosis.) The diffraction band at the edge of the cell gave a mean of 0·714 μ wide, with standard error 0·041 in 128 measurements on 32 photographs. Measurements were made from the outer edge of this band and then corrected to give the dimensions as from the true edge. This was 0·606 × the width of the band out from the inside of the band. The correction was based upon the formula of Ponder and Millar (1924): $T_0/T_1 = N_1/N_0$. Here, N_0 and N_1 are the refractive indices of the media (air = 1·0) and the cell, using the value of 1·54 for dried protein as an approximation to that of the fixed cell; T_0 is the distance from the inner edge of the diffraction band to the true cell edge, and T_1 is the distance from the true edge of the cell to the outer edge of the band.

RESULTS.

Haemoglobin (*Table* 1).

Consideration of the regression lines indicates that over the whole range the variation is only about 15 p.c. Since the mean ages were so close it was decided to compare the means directly by an analysis of variance, ignoring the minor

TABLE 1.

Haemoglobin all ages.

	Number	Mean	Variance	Variance allowing for Regression	Standard error	Mean age	Variance of ages	Linear Regression equation	Signif.	Quadratic Regression equation	Signif.	Signif. of diff. between Linear and Quadratic Regression
Aboriginal males	43	16·42359	1·477143	1·265045	0·1715	36·3023	372·5	H = 15·6794 + +0·02048A	•	H = 14·4383 + +0·08374A − −0·000799A²	•	N.S.
European males	73	15·91811	1·108303	1·133468	0·1295	33·8356	99·7	H = 15·8031 + +0·003448A	N.S.	H = 14·9397 + +0·05263A − −0·000700A²	N.S.	N.S.
Aboriginal females	57	16·21657	2·020662	1·784066	0·1883	27·2982	208·4	H = 15·3103 + +0·03316A	••	H = 13·9542 + +0·1148A − −0·0117A²	•	N.S.
European females	83	14·10311	0·774818	0·659947	0·0966	31·5060	170·6	H = 13·4856 + +0·01960A	••	H = 11·3409 + +0·09054A − −0·000914A²	•••	••

Where H = haemoglobin concentration in gms. per 100 ml. of blood
A = age in years

N.S. = not significant ·05< P
• = ·01<P<·05
•• = ·001<P<·01
••• = P<·001
} These symbols apply to all Tables.

differences due to regression. Pooling all observations gave a grand variance of 1·14377 with 244 degrees of freedom and a grand standard error of 0·0685. To eliminate some avoidable error, the sums of squares used in the calculations were the residuals, which did not contain that part caused by regression.

The variance between aboriginal men and women was not significant ($F = 1·09$ on 244 and 1 d.f.) while that between European men and women was very significant ($F = 112$ on 1 and 244 d.f., $P < ·001$). The variance between European and aboriginal males was just significant ($F = 5·91$ on 1 and 244 d.f., $·01 < P < ·05$), but that between the females was very significant ($F = 131$ on 1 and 244 d.f., $P < ·001$). The ratio of the variances between men and women in the two groups was just not significant ($F = 122$ on 1 and 1 d.f., $P \doteqdot ·06$).

When the values were analysed into age groups (Table 2), it was found that children less than 16 years old had lower haemoglobin values than adults. There were no significant changes in either group of women during their reproductive life; however, postmenopausal European women showed a significant rise, but aboriginal women did not. Neither group of men over 50 years showed any significant change. The young of all groups and the older European women were therefore excluded and the data reanalysed.

The means shown in Table 2 for 16-50 years old European females, 16-80 years old aboriginal females and 16-80 years old men were used, with the original grand variance. The variance between the men was very significant ($F = 12·9$ on 1 and 244 d.f., $P < ·001$), as was that between the women ($F = 151$ on 1 and 244 d.f., $P < ·001$). The variance between the sexes was not significant for aborigines ($F = 3·65$ on 244 and 1 d.f.) but for Europeans it was very significant ($F = 117$ on 1 and 244 d.f., $P < ·001$). The ratio "F" of the variances between sexes in the two groups was significant ($F = 426$ on 1 and 1 d.f., $·01 < P < ·05$) while that between Europeans and aborigines in the two sexes was not ($F = 13·4$ on 1 and 1 d.f.).

Beyond recording the values obtained for the linear and quadratic regression coefficients and equations, and their significance, the relatively small numbers did not justify further analysis. But sufficient data have been included to allow this to be done if future surveys produce enough additional information.

It is of interest to note that all the regression equations rise to a maximum at about 45 years and then, having a negative second derivative, fall off once more, giving lower values at both extremes of life. As these equations are only of second degree they would not disclose any minor fall during the fertile period in women. The aboriginal linear regression coefficients are positive because of the inclusion of prepubertal individuals. That of the European females is also positive because of the postmenopausal rise, but the European male shows neither of these influences and his coefficient is not significantly different from zero.

TABLE 2.

Haemoglobin in age groups.

Aboriginal males			European males		Aboriginal females			European females		
Age	Number	Mean	Number	Mean	Number	Mean		Number	Mean	
0-15	6	15·25633	—		11	15·10544		—		
16-30	28	16·64179	69	15·90333	29	16·23738	16·48940	43	13·97475	14·01142
31-50					12	17·09859		31	14·06230	
		16·64904		15·91811			16·48228			
51-80	9	16·52300	4	16·17300	5	16·42359		9	14·74510	

"t" tests of significance:—

Males: aboriginal
16-50 against 0-15 difference 1·38546 $t_{28} = 2·4587^{*}$
51-80 against 16-50 difference 0·11879 $t_{33} = 0·2435$ N.S.

European
51-80 against 16-50 difference 0·26967 $t_{71} = 0·4916$ N.S.

Females: aboriginal
0-15 against 16-50 difference 1·38396 $t_{38} = 2·822^{**}$
31-50 against 16-30 difference 0·86121 $t_{39} = 1·7215$ N.S.
51-80 against 16-50 difference 0·06581 $t_{44} = 0·0954$ N.S.

European
31-50 against 16-30 difference 0·08755 $t_{73} = 0·4165$ N.S.
51-80 against 16-50 difference 0·73439 $t_{61} = 2·3351^{*}$

TABLE 3.

Erythrocyte counts (in millions).

	Number	Mean	Variance	Standard error	Mean age	Variance of ages	Linear Regression equation	Signif.	Quadratic Regression equation	Signif.	Signif. diff. between Linear and Quadratic Regression
Aboriginal males	39	5·1088	·786467	·142039	39·4102	311·2	$R = 5·3361 - -·005768A$	N.S.	$R = 5·2126 + +·01399A - -·00006739A^{2}$	N.S.	N.S.
European males	48	4·8888	·295877	·078512	35·3125	147·2	$R = 4·5621 + +·009250A$	N.S.	$R = 5·3372 - -·02994A + +·0004895A^{2}$	N.S.	N.S.
Aboriginal females	47	4·9152	·510590	·104238	26·0213	182·2	$R = 5·0242 - -·004188A$	N.S.	$R = 4·3390 + +·04495A - -·0008779A^{2}$	N.S.	N.S.
European females	57	4·5017	·260284	·067575	30·0175	150·4	$R = 4·8266 - -·010820A$	N.S.	$R = 5·2042 - -·03354A + +·0003373A^{2}$	N.S.	N.S.

where R = erythrocyte count per cubic mm.

Erythrocyte Count (*Table* 3).

Neither the linear nor the quadratic regression lines are significant. Analyses of variance were carried out on the means of each group, with a grand variance of 0·444918 with 187 degrees of freedom and standard error of 0·0487. The variance between sexes in the aborigines was not significant ($F = 1·81$ on 1 and 187 d.f.) but that in Europeans was quite significant ($F = 8·77$ on 1 and 187 d.f., $·001 < P < ·01$). The variance between aboriginal and European males was not significant ($F = 1·83$ on 1 and 187 d.f.), that between the females was quite significant ($F = 11·1$ on 1 and 187 d.f., $·001 < P < ·01$). The ratio of the variance between sexes in aborigines and Europeans was not significant ($F = 4·86$ on 1 and 1 d.f.), but the difference is in the same direction as that of the haemoglobins, confirming the latter. The ratio of the variances between groups in the two sexes was not significant ($F = 6·05$ on 1 and 1 d.f.).

Mean Corpuscular Haemoglobin (*Table* 4).

The grand variance was 24·7738 with 182 degrees of freedom, and the grand standard error was 0·367. The variance between the sexes was not significant for either aborigines ($F = 50·8$ on 182 and 1 d.f.) or Europeans ($F = 2·57$ on 1 and 182 d.f.). The variance between European and aboriginal women was not significant ($F = 3·08$ on 1 and 182 d.f.). That between the men was significantly close ($F = 1810$ on 182 and 1 d.f., $·01 < P < ·05$) but this is probably only accidental. The ratio of the variances between sexes in aborigines and Europeans was not significant ($F = 131$ on 1 and 1 d.f.), but that of variance between aborigines and Europeans in the two sexes was quite significant ($F = 5582$ on 1 and 1 d.f., $·001 < P < ·01$). The two groups of women have significant, but not significantly different, linear regressions, and non-significant quadratic regressions. The men's regression lines are not significant.

Erythrocyte Diameter (*Table* 5).

An analysis of variance of the means was performed, neglecting the relatively slight regressions. The grand variance was 0·139535 on 229 degrees of freedom (eliminating that part due to regression), the grand standard error was 0·0241.

The variance between sexes was not significant for aborigines ($F = 46·5$ on 229 and 1 d.f.) but just significant for Europeans ($F = 5·45$ on 1 and 229 d.f., $·01 < P < ·05$). There was a very significant difference between aboriginal and European males ($F = 18·4$ on 1 and 229 d.f., $P < ·001$) and a just significant one between the females ($F = 6·28$ on 1 and 229 d.f., $·01 < P < ·05$). The ratio of variances between sexes in aborigines and Europeans was just significant ($F = 253$ on 1 and 1 d.f., $·01 < P < ·05$) but that between aborigines and Europeans in the sexes was not ($F = 2·9$ on 1 and 1 d.f.). The aboriginal males and females both show a slight increase in diameter with age while the Europeans do not.

TABLE 4.

Mean corpuscular haemoglobin.

	Number	Mean	Variance	Standard error	Mean age	Variance of ages	Linear Regression equation	Signif.	Quadratic Regression equation	Signif.	Signif. diff. between Linear and Quadratic Regression
Aboriginal males	38	32·907	48·67037	1·132	39·5000	319·7	H = 30·818 + +0·0529A	N.S.	H = 25·347 + +0·2949A − −0·002113A²	N.S.	N.S.
White males	47	32·882	17·11984	0·604	34·4468	113·8	H = 34·508 − −0·0472A	N.S.	H = 28·680 + +0·2545A − −0·004223A²	N.S.	N.S.
Aboriginal females	44	33·062	25·58112	0·762	26·4091	180·6	H = 29·463 + +0·1363A	•	H = 31·523 + +0·00798A + +0·001900A²	•	N.S.
White females	57	31·310	15·56602	0·523	30·0175	150·3	H = 28·257 + +0·1017A	•	H = 27·740 + +0·1328A − −0·0004619A²	N.S.	N.S.

where H = mean corpuscular haemoglobin in $\mu\mu$.

TABLE 5.

Erythrocyte diameters.

	Number	Mean diameter	Variance	Standard error	Mean age	Variance of ages	Linear Regression equation	Signif.	Quadratic Regression equation	Signif.	Signif. diff. between Linear and Quadratic Regression
Aboriginal males	41	7·5950	0·08974199	0·04678	32·0732	353·8	D = 7·3433 + + ·0078435A	••	D = 7·2689 + + ·012009A − − ·00005769A²	•	N.S.
European males	64	7·9157	0·1507658	0·04854	34·4219	97·3	D = 7·5990 + + ·0091680A	N.S.	D = 7·9878 − − ·012577A + + ·0003052A²	N.S.	N.S.
Aboriginal females	60	7·6061	0·10030231	0·04089	24·5833	198·9	D = 7·4459 + + ·0066446A	•	D = 6·8750 + + ·045284A − − ·0006346A²	•••	•••
European females	76	7·7678	0·17415118	0·04787	30·8158	168·5	D = 7·7277 − − ·0012942A	N.S.	D = 7·8155 − − ·0017188A + + ·000005462A²	N.S.	N.S.

where D = mean diameter in μ of individual's cells (50 measured for each individual).

To reach an estimate of the non-circularity and anisocytosis in each group a further analysis of variance was performed (Table 6). The variance within cells represents the squares of the differences between major and minor diameters of the cells (after allowing for the degrees of freedom), and thus the non-circularity—no distinction has been made between oval and tear-drop shapes. The variance between cells represents the squares of the differences between the mean diameters of the cells (after allowing for the degrees of freedom), and thus the anisocytosis. The variances were compared by means of "z tests".

Anisocytosis. The difference between the sexes was just significant in the aborigines ($z = 0{\cdot}0676$ on 2940 and 2009 d.f., $\cdot 01 < P < \cdot 05$) and very significant in Europeans ($z = 0{\cdot}157$ on 3724 and 3136 d.f., $P < \cdot 001$). The difference between European and aboriginal men was significant ($z = 0{\cdot}0953$ on 2009 and 3136 d.f., $\cdot 001 < P < \cdot 01$) but the difference between the women was not ($z = 0{\cdot}00571$ on 2940 and 3724 d.f.). The ratio between groups of the differences between sexes was not significant.

Non-circularity. The sex difference for aborigines was not significant ($z = 0{\cdot}0193$ on 3000 and 2050 d.f.) but that for Europeans was just significant ($z = 0{\cdot}0591$ on 3800 and 3200 d.f., $\cdot 01 < P < \cdot 05$). The difference between the two groups of men was not significant ($z = 0{\cdot}0417$ on 2050 and 3200 d.f.) nor was that between the women ($z = 0{\cdot}00199$ on 3000 and 3800 d.f.).

Eccentricity. The root-mean-square average of the differences between major and minor diameters of the cells can be obtained by taking the square root of the variances used as estimate of non-circularity. Using the mean diameters of the four different groups permits one to calculate the eccentricity "e" from the formula $e = \sqrt{1 - b^2/a^2}$, where "a" and "b" are the major and minor diameters. The results are shown in Table 7. Here, "a" and "b" are found from the mean diameter, and from the difference a-b (= R.M.S.). The grand eccentricity, found by weighting the group eccentricities by the degrees of freedom of each, is 0·446, based on 12,050 cells. But it must be emphasized that this figure refers only to the condition of dried, fixed and stained cells. Concerning stained films, Ponder and Saslow (1928, p. 325) say, " . . . it is better to think of a circular cell as a special form of distortion". However, as estimates of non-circularity are usually based on dried films it seemed useful to include this record.

DISCUSSION.

Mean haemoglobin values of 15·9 gm. and 14·1 gm. per 100 ml. for European men and women respectively are not significantly different from Wintrobe's (1956) figures of 16·0 gm. and 14·0 gm. compiled from many sources. Other values for Europeans in Australia are 15·4 gm. and 13·7 gm. (McLean, 1938), 16·2 gm. for 77 men in Sydney (Wardlaw, 1941), and 15·43 gm. and 13·93 gm. in Melbourne (Bryce and Jakobowicz, 1943). All these workers used the

TABLE 6.

Erythrocyte Diameters—parts of analysis of Variance.

	Variance between individuals	Residual Variance between individuals omitting Regression	Mean Square Within individuals	*Non-circularity* Variance within cells for all individuals	*Anisocytosis* Mean Square between cells for all individuals	Variance of Means of cells for all individuals	Total Variance
Aboriginal males	0·1074487(40)	0·0897422(38)	0·697534(4059)	0·736935(2050)	0·454134(2009)	0·227067(2009)	0·795412(4009)
European males	0·1550673(63)	0·150766(61)	0·420642(6336)	0·608186(3200)	0·292731(3136)	0·146366(3136)	0·569174(6399)
Aboriginal females	0·12810503(59)	0·100302(57)	0·713697(5940)	0·805534(3000)	0·619986(2940)	0·309993(2940)	0·832668(5999)
European females	0·1698189(75)	0·174151(73)	0·702077(7524)	0·798229(3800)	0·603925(3724)	0·301963(3724)	0·862705(7599)

Numbers in brackets give the Degrees of Freedom.

TABLE 7.

Eccentricity of the erythrocytes.

	Variance within cells	Standard Deviation = R.M.S.	Degrees of Freedom	Standard Error of Standard Deviation	Mean diameter	"a"	"b"	Eccentricity "e"
Aboriginal males	0·73693	0·8584	2050	0·01341	7·5950	8·0242	7·1658	0·447
European males	0·60819	0·7799	3200	0·009788	7·9157	8·3056	7·5257	0·424
Aboriginal females	0·80553	0·8975	3000	0·01159	7·6061	8·0548	7·1573	0·458
European females	0·79823	0·8934	3800	0·01025	7·7678	8·2145	7·3211	0·453

oxygen capacity method which is 3 p.c. lower than the iron analysis standard (Walsh, 1953). Lancaster and Banks (1957) found values of 16·2 gm. for 37 men and 14·2 gm. for 63 women in Sydney. The largest Australian survey so far is that by Walsh *et al.* (1953) in New South Wales. They obtained a mean of 15·76 gm. ± ·025 for 1645 men and 13·93 gm. ± ·032 for 1142 women of various ages. Evidently, although haemoglobin values are fairly high, estimations by different observers upon Europeans in Australia vary only a little and the present series may be considered representative.

For aborigines, Wardlaw (1935) found a mean haemoglobin of 15·2 gm. in 25 male natives around Haast's Bluff. He also showed that 9 "wild" natives had a mean 0·5 gm. less than mission natives (16·4 gm.) who probably enjoyed a better diet. Walsh (personal communication) obtained a figure of 15·0 gm. for 34 males at Darwin. In the Warburton Range Davis and Pitney (1957) found a mean of 16·2 gm. but their samples appeared to have increased by 1 gm. between the field and the laboratory, so the true mean is probably lower. The Warburton natives showed no gross malnutrition, but their living conditions were probably harder than those of the Haast's Bluff natives. The present figure of 16·4 gm. for male aborigines is in line with these other findings.

The difference of 0·5 gm. between aboriginal and European men in this study may reflect the difference in altitude between Haast's Bluff and Adelaide. Haast's Bluff Settlement is about 1900 feet above sea level, Adelaide only 100 feet; there was a mean difference in barometric pressure of 49·5 mm. mercury, estimated over one year (Adelaide Weather Bureau, 1956). Such a difference could be present in the women, too, but obscured by differences in diet, menstrual loss, and hormone levels.

The level for aboriginal women was 16·2 gm. which is only 0·2 gm. below the males, compared with a deficit of 1·8 gm. for European women. This is not in agreement with Walsh's (1957, personal communication) figure of 13·2 gm. for 35 female aborigines at Darwin—1·8 gm. lower than the males. Davis and Pitney (1957) found a value of 14·5 gm. for aboriginal women but, as mentioned above, this may be to 1 gm. too high. The present case is one of the few recorded in which women's haemoglobin almost equals that of men. It is possible that better food, lower menstrual loss, some dehydration or hormonal differences are responsible.

Reasons for the "normally" existing sex difference are not clear; but there are three major views. One is that the higher figure for males reflects their greater muscular development and activity (Schnitter, 1946). However, Adcock *et al.* (1949) found that sedentary workers had higher haemoglobin values than footguards on the same diet, while Berry (1949) found a wide range with a mean of 16·0 gm. in Olympic athletes. The second view postulates differences in B.M.R. produced by the different sex hormones (Steinglass *et al.*, 1941). In rats, man and other animals testosterone increases the haemoglobin value and

erythrocyte counts, while oestrogens lower them (Taber *et al.*, 1942; Gordon and Charipper, 1947; Daughaday *et al.*, 1948). The post-middle age fall in men's haemoglobin and the rise in women's (Olhrich, 1947; Shapleigh *et al.*, 1952; Jefferson *et al.*, 1953; Hawkins *et al.*, 1954) have been attributed to falling hormone levels. But the rise in women could be due to cessation of menstrual loss. The third view is that the sex difference is due simply to menstrual loss in women. After puberty the level in boys continues to rise while that in girls may rise slightly, remain stationary or fall (Davidson *et al.*, 1935; Fullerton, 1936; Leichsenring *et al.*, 1941; M.R.C., 1943; Millar, 1952; Hawkins *et al.*, 1954). These changes could be hormonal, however. Different workers record different effects after the menopause. Some find a rise (Davidson *et al.*, 1935; Fullerton, 1936; Bryce and Jakobowicz, 1943; M.R.C., 1943; Millar, 1952) as in the European women in this series. Others can detect no change (Olhrich, 1947; Shapleigh *et al.*, 1952; Jefferson *et al.*, 1953; Walsh *et al.*, 1953; Hawkins *et al.*, 1954) as in the aboriginal women reported here. Wintrobe *et al.* (1935) found no sex difference in non-menstruating mammals and are supported by Creshoff *et al.* (1949) for rats. On the other hand, Steinglass *et al.* (1941) found a distinct difference in rats and quote other workers on other animals in their support. Garry *et al.* (1954) feel that the issue is still undecided.

Widdowson and McCance (1936) fed 100 mg. iron a day to men and women. The men showed little increase in haemoglobin, but the women's levels rose to those of the men. This has been confirmed by Sankaran and Rajogopal (1938), but O'Reilly (1948) and Garry *et al.* (1954) could not demonstrate this. W.A.A.F.'s increased their haemoglobin on the high iron army ration (M.R.C., 1943, and Yudkin, 1944). Many writers have doubted if menstrual losses are large enough to cause iron deficiency, but Widdowson and McCance (1936) and Barer and Fowler (1936) showed that they might well be. Still, male blood donors may lose roughly equivalent amounts without a fall in haemoglobin (Bryce and Jakobowicz, 1943; and Walsh, 1953). It seems likely that the sex difference is due to both a hormonal difference and a relative iron deficiency, but iron becomes of significance only when the intake is quite low or very high. So far as aboriginal women are concerned, it is generally agreed that the duration of menstrual flow is short and the loss scanty (Cleland, personal communication; Poidevin, 1957; and others). This, together with a possibly high iron content in the diet, may explain their high haemoglobin level. The possibility of some underlying hormonal peculiarity, leading to a low loss, and a high haemoglobin, must also be kept in mind.

Erythrocyte counts for Europeans in Australia were significantly lower than those given by Wintrobe (1956), i.e. 4·9 million per cu.mm. for men and 4·5 for women as against $5{\cdot}4 \pm 0{\cdot}8$ and $4{\cdot}8 \pm 0{\cdot}6$ respectively. However, Lancaster and Banks (1957) also obtained low figures for white Australians in Sydney (4·7 for men and 4·4 for women) and it may be that Europeans in

Australia have lower counts than Europeans reported elsewhere. In the present study, however, the lower values could also be due to adsorption of cells on to the glass, or to lysis. It was unfortunate that conditions precluded the employment of more satisfactory methods. Nevertheless, the values relative to those of the control series are of interest since they confirm the haemoglobin finding so far as the greater error in their estimation will permit.

Mean corpuscular haemoglobins for Europeans here (32·88 and 31·31) are higher than Wintrobe's (29 ± 2 and 29 ± 2) but closer to those of Lancaster and Banks for Sydney (33·2 and 32·4). These authors ascribe their higher figures to the iron assay method of standardizing haemoglobin which gives results greater than the old oxygen capacity standards. Another factor, which they do not mention, is the lower erythrocyte counts they obtained. The aboriginal values here were not significantly different from those of Europeans, although the ratio of the variance between the two groups of women over that of the two groups of men is quite significant. This shows that the higher haemoglobin in the aboriginal women is partly due to higher M.C.H. as well as to the higher erythrocyte count *vis-à-vis* European women as compared with the corresponding men.

Interpretation of studies on erythrocyte diameters has always proved difficult. The variations between cells in plasma and in fixed stained films, between methods of measurement, conditions and individuals have all caused some confusion. Ponder and Millar (1924) obtained means of 8·8 μ for cells in plasma and 7·6 μ for dried cells. Other workers have varied in their figures for dried stained cells according to their methods and the point in the marginal diffraction band that they took as the true edge (Ponder and Saslow, 1928; Leichsenring *et al.*, 1944). Ponder and Saslow (1930) suggested that means should not be considered significantly different unless their difference was 5 × their standard error instead of 2 ×. Statistically that is rather unsound, and it is unnecessary in comparing groups of people if a sufficient number of individuals is taken or if sufficient replications are performed. As the present series included 101 aborigines and 140 Europeans it was felt that 50 cells, measured along maximum and minimum diameters, sufficed. The mean diameter obtained in this way is possibly not as good an estimate of the "true" mean diameter of the cell as that obtained by the area method of Hynes and Martin (1936) but it permits calculation of the non-circularity and eccentricity of the cells.

For dried cells Wintrobe (1930) quotes the values of various authors as 7·42, 7·7, 7·9, 7·55, 7·3 and 7·2 μ. The present values are at the upper level of this series. Adding 0·8 μ (Wintrobe, 1956) gives figures for cells in plasma close to those of Ponder. The European males were 0·14 μ larger than the females. Wintrobe (1930, 1956) gives the reverse. Price-Jones (1929) found no difference. The aborigines here showed no sex differences, so its significance

is dubious. The Europeans had larger cells than aborigines of the same sex but this might be an altitude effect. Smith *et al.* (1924), Wintrobe (1930), and Radhakrishna Rao (1938) all report a decrease in diameter at higher altitudes. This may be due to a gaseous alkalosis from hyperventilation. However, during acclimatization there is a temporary increase in cell diameter because of a reticulocytosis (Wintrobe, 1930, 1956). A significant increase in diameter with age was found in the aborigines but not in the Europeans (perhaps because of lack of European controls in the extremes of life). Olhrich (1947) also found such an increase but Price-Jones (1921) found no increase in diameter nor did Shapleigh *et al.* (1952) in M.C.V.

The estimates of anisocytosis disclosed a considerably greater incidence in women than in men. Also, the aboriginal men showed more than European men. This may be a reflection of greater haemopoesis—because of menstruation in women, and of the higher altitude in the case of aboriginal men. The variance between individual means is not due to anisocytosis but to differences of drying, etc., of the films and to true individual differences. These variances are of the same order of magnitude as those found by other investigators. The variances within cells, being a measure of their non-circularity, show only one significant difference, namely, that between European men and women. This, too, may well be a result of the greater haemopoesis in women.

The eccentricity found from these figures (0·45) is very similar to Niemann's (1930) figure of 0·46 and Olhrich's (1947) 0·45. This indicates that the normally stained human erythrocyte is not a circle but an ellipse, as in all other vertebrates. This is contrary to generally accepted ideas, but it is not surprising that it usually escapes notice since Niemann states that the eye is incapable of detecting eccentricities of $e < 0{\cdot}47$.

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SUMMARY.

This paper records haemoglobin values, erythrocyte counts, mean corpuscular haemoglobin values, erythrocyte diameters, and estimates of anisocytosis, non-circularity and mean eccentricity of erythrocytes for a group of Central Australian aborigines as compared with a corresponding group of Adelaide European controls.

Haemoglobin values in aboriginal males were slightly greater than in Euro-

pean controls. Those in aboriginal females are markedly greater than in European females. Possible explanations are discussed.

Erythrocyte counts revealed similar but less marked differences. European figures were lower than those accepted elsewhere, but were close to some reported from Sydney.

Mean corpuscular haemoglobin values for the men were not different but the aboriginal women had slightly higher values than European women. The Europeans had slightly higher values than most recorded elsewhere, but conformed to the Sydney findings.

Erythrocyte diameters disclosed no significant sex difference in the aborigines but were slightly greater in European men than women. Aboriginal males showed smaller diameters than European males while those in the aboriginal females were only slightly smaller than in European females. The aborigines showed an increase in diameter with age.

Anisocytosis was estimated from the variance between cells. The two female groups showed about the same amount but the aboriginal men showed slightly, and European men considerably, less.

Non-circularity was estimated from the variance within cells. European men showed less than European women, other differences were not significant.

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THE EFFECT OF URANIUM ACETATE ON HEPARIN INDUCED LIPEMIA CLEARING

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It has been demonstrated that uranium salts elevate the serum lipids under a number of different circumstances (Heymann and Clark, 1945; Day *et al.*, 1957a). This hyperlipemia was attributed to the nephrotoxic action of uranium and it was shown that bilateral nephrectomy resulted in similar lipid changes (Heymann and Clark, 1945). Recently evidence has been presented that circulating heparin plays a part in lipid transport (Brown, 1952; Spitzer, 1953; Bragdon and Havel, 1954; Day *et al.*, 1956, 1957b, 1957c) and lipemia clearing activity has been demonstrated in normal plasma (Robinson *et al.*, 1954; Engelberg, 1955). Since uranium acetate produced its most marked effect on the total fatty acids and since clearing factor can be considered to have primarily a lipolytic action (Shore *et al.*, 1953), the possibility arose that uranium hyperlipemia or indeed nephrotic hyperlipemia might be explicable on the basis of inhibition of clearing factor activity. It might be noted in this regard that the surface active agent Triton, which has been shown to produce hyperlipemia (Kellner *et al.*, 1951), has been also shown to inhibit lipemia clearing factor (Brown *et al.*, 1953; Schotz *et al.*, 1957). The inhibitory effect of uranium acetate on heparin-induced lipemia clearing factor was therefore determined in order to analyse the pathogenesis of uranium hyperlipemia and also to obtain basic information regarding the mechanism of the clearing reaction. In this paper, therefore, the alteration in heparin-induced lipemia clearing occurring in uranium acetate treated rabbits is investigated. This change is described both with regard to the clearing factor elaborated under these circumstances and, since heparin has been shown to reduce serum lipids in rabbits (Day *et al.*, 1957c, 1957d), with regard to the fall in serum lipids produced.

METHODS.

Experiment I: Twelve normally fed mongrel rabbits of mixed sexes varying in weight from 1·37 kg. to 2·05 kg. were randomised into two equal groups. To one group a single subcutaneous injection of 7·5 mg./kg. of uranium acetate was administered, while to the other (control) group a corresponding volume of saline was given. Ten days later each rabbit was given intravenously 1000 units (10 mg.) of heparin, blood samples being col-

lected immediately prior to injection and 15 minutes after injection. The lipemia clearing factor elaborated as a result of heparin injection was determined in both groups.

Experiment II: Fourteen rabbits (1·71-2·90 kg.) were randomised into two groups of eight and six rabbits respectively. To the first group of eight animals a single subcutaneous injection of 5·0 mg./kg. of uranium acetate was administered. The second group served as controls. Ten days later 1000 units of heparin were administered and the effect determined on the elaboration of lipemia clearing factor in the two groups. Plasma cholesterol, phospholipid and total esterified fatty acids were determined prior to commencement of the experiment, and also before and after the heparin injection 10 days later.

Experiment III: Four of the control animals used in Experiment II were used a second time. Each was given an intravenous injection of 5 mg./kg. of uranium acetate and 30 minutes later a blood sample taken, 1000 units of heparin administered and a further sample taken 15 minutes after the heparin. Lipemia clearing factor elaborated under these circumstances was determined.

Experiment IV: Fourteen rabbits (1·76-2·44 kg.) were fed a cholesterol supplemented diet containing daily 1 gm. cholesterol and 6 ml. peanut oil in 75 gm. rabbit mash. After seven days cholesterol feeding, plasma cholesterol, phospholipid and total fatty acids were determined and on the basis of these levels the rabbits were randomised into two groups of eight and six respectively. The first group was given 5 mg./kg. uranium acetate subcutaneously, while the second group was given an equivalent saline injection. The cholesterol feeding was continued for another 10 days when each rabbit was given 1000 units of heparin and blood samples taken before and 15 minutes after the injection. Plasma lipid levels and lipemia clearing factor were determined in each sample.

Clearing factor activity. The determination of lipemia clearing factor was based on the method described by Grossman (1954). Blood samples were taken directly into chilled oxalated centrifuge tubes and kept at 5° C. during centrifugation (2,500 r.p.m. for 15 mins.) and subsequently until the determinations were performed. The emulsion substrate used was an homogenised Tween "60" stabilised preparation consisting of 10 gm. maize oil, 0·5 gm. Tween "60" and 5 gm. glucose per 100 ml. distilled water. This emulsion was diluted 1:20 with water prior to use. The actual test was performed by bringing 0·5 ml. plasma to 27° C. and adding 0·1 ml. diluted emulsion substrate, mixing and reading the optical density in 2 mm. cells in a Unicam S.P. 600 spectrophotometer at 650 mμ. The optical density was read immediately after mixing and subsequently at 5, 10, 20, 30, 45 and 60 minutes. In each case the clearing activity in both pre- and post-heparin samples was determined under identical conditions.

Plasma lipids. Plasma lipids were all determined after extraction with a 3:1 alcohol:ether mixture using a special vibrator pipette (Brown, 1954) to aid dispersion. For cholesterol determinations an aliquot quantity of the extract was evaporated to dryness in a water bath at 70° C. and the residue taken up in 6 ml. glacial acetic acid (B.D.H. AnalaR). The cholesterol was then determined using the method of Zlatkis, Zak and Boyle (1953) with the volume of reagents doubled. When unextracted serum or plasma were used directly for cholesterol determinations the values were higher than with extracted serum or plasma and this divergency was particularly apparent with the rabbit plasma. Other substances than cholesterol present in plasma contributed to the colour development and the use of extracted plasma overcame this difficulty with its attendant higher values. Extraction also produced a more marked spectral absorption peak at 560 mμ than was the case with unextracted serum or plasma. For these reasons it was decided to use an alcohol-ether extract instead of the plasma directly, but to retain the Zlatkis, Zak and Boyle method with its attendant simplicity.

Phospolipid was determined as lipid phosphorus (Brown, 1954) after evaporation of an aliquot quantity of the above extract. Total esterified fatty acids were determined on

the same extract by the method of Stern and Shapiro (1953). When lipemic plasma was used for fatty acid determinations turbidity developed following the addition of the NaOH. For the cholesterol-fed rabbit plasma, therefore, the fatty acid method was further modified, alcoholic:aqueous NaOH being substituted for aqueous and this was neutralised by alcoholic: aqueous HCl. Following colour development 1 ml. of ether was added and the colour read at 520 mμ as usual.

RESULTS.

The effect of uranium acetate on lipemia clearing factor.

Prior treatment of rabbits with uranium acetate prevents the subsequent elaboration by heparin of clearing factor. Fig. 1 demonstrates this inhibiting effect of uranium acetate and combines the results of Experiments I and II. The mean change in optical density is plotted against the time. There is marked clearing activity in the control series (the average curve of 12 control rabbits from both experiments is shown) when the post-heparin plasma is compared with the pre-heparin plasma. In the uranium acetate group, however (average curves of the seven rabbits surviving), there is little clearing activity and the mean post-heparin curve approaches that of the mean pre-heparin curve for the same animals.

In order to exclude an *in vitro* inhibitory effect of uranium acetate on clearing, the activity of five of the control post-heparin plasmas was determined a second time after incubation with 0·1 mg. (0·1 ml.) uranium acetate. The mean curve obtained under these circumstances is shown also in Fig. 1. Clearing activity is present at the same level as in the control without *in vitro* uranium added. The inhibitory effect of uranium acetate on clearing factor is not therefore due to any direct chemical inhibition.

The effect of intravenous uranium acetate 30 minutes prior to heparin on the consequent elaboration of clearing factor is shown in Fig. 2. The mean curve of the four animals treated demonstrates that uranium acetate does not inhibit lipemia clearing activity under these circumstances. It would seem, therefore, that the inhibitory effect of uranium acetate on heparin-induced lipemia clearing is due to a secondary effect on the animal, either destruction of the site of clearing factor production, or the elaboration of clearing factor inhibitors.

Fig. 3 shows the inhibitory effect of uranium acetate administration on heparin-induced lipemia clearing in cholesterol-fed rabbits. The individual behaviour of each of the six control animals and of the seven uranium acetate animals surviving in Experiment IV is plotted against time in this Fig. The pre-heparin curves are not included. It is apparent that there is no clearing factor activity in any of the uranium acetate treated animals, but activity is present in all of the six controls. It might be noted also from Fig. 3 that in the three controls in which gross plasma lipemia exists, there is a temporary inhibition of activity. This feature of the more lipemic plasma is overcome by

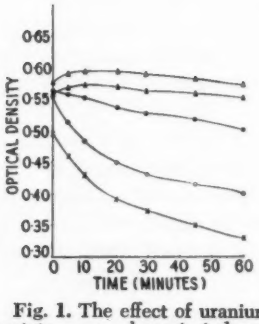

Fig. 1. The effect of uranium acetate on heparin-induced lipemia clearing activity in normally fed rabbits.
○—○ Control, post-heparin.
△—△ Control, pre-heparin.
●—● Uranium acetate (10 days before), post-heparin.
▲—▲ Uranium acetate (10 days before) pre-heparin.
×—× Control, post-heparin plus *in vitro* uranium acetate.
Details in text.

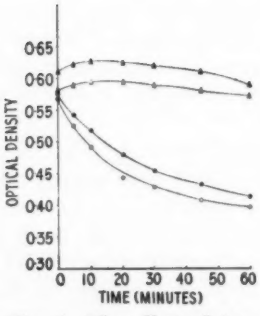

Fig. 2. The effect of intravenous uranium acetate on heparin-induced lipemia clearing activity. Uranium administered 30 minutes prior to heparin.
○—○ Control, post-heparin.
△—△ Control, pre-heparin.
●—● Uranium acetate, post-heparin.
▲—▲ Uranium acetate, pre-heparin.
Details in text.

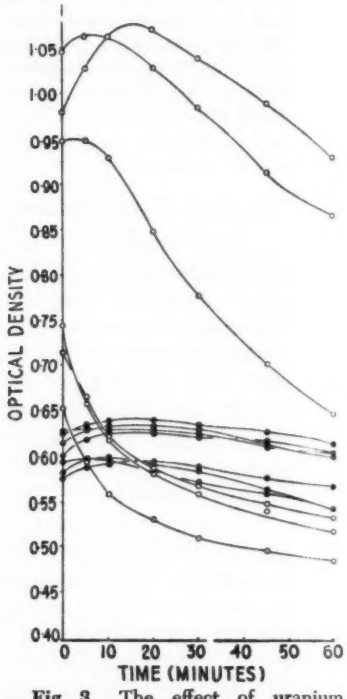

Fig. 3. The effect of uranium acetate on heparin-induced lipemia clearing in cholesterol fed rabbits.
○—○ Control, post-heparin.
●—● Uranium acetate, post-heparin.
Details in text.

the 10-20 minute reading and clearing activity is apparent after this point. The three controls where lipemia is not marked behaved with respect to clearing activity in just the same way as the normally fed controls.

The effect of uranium acetate on the serum lipids.

The rise in total fatty acids, cholesterol and phospholipids produced following uranium acetate administration to normally fed rabbits is shown in Fig. 4.

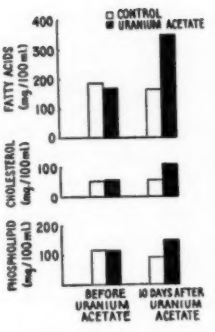

Fig. 4. The effect of uranium acetate on serum lipids in normally fed rabbits. Details in text.

The level of these three lipid fractions prior to the injection of the uranium acetate and 10 days after the injection is illustrated in this graph. The mean total fatty acid level rose in the treated group from 166 (± 12·3)[1] mg./100 ml. to 348 (± 31·3) mg./100 ml. The corresponding control levels were 188 (± 37·6) to 162 (± 12·7) at 0 and 10 days respectively. The cholesterol levels in the uranium acetate treated group were increased from 52 (± 10·3) mg./100 ml to 107 (± 27·5) mg./100 ml. compared with 50 (± 10·9) mg./100 ml. and 54 (± 12·0) mg./100 ml. in the control group. The phospholipid levels were also increased by uranium acetate treatment from 113 (± 9·1) mg./100 ml. to 148 (± 17·0) mg/100 ml. compared with 117 (± 20·9) mg./100 ml. and 92 (± 12·2) mg./100 ml. in the control. Thus uranium acetate produced significant increases in the lipid fractions studied in normally fed rabbits.

In the cholesterol fed rabbits investigated, uranium acetate produced greater increases in total fatty acids and phospholipid when compared with the control, but in the case of cholesterol this fraction was increased approximately to the same extent in each group. These findings confirm earlier reports (Day *et al.*, 1957a) for cholesterol fed rabbits and will not be considered further in this paper.

The effect of heparin on the serum lipids in uranium acetate treated rabbits.

Fig. 5 demonstrates the effect of heparin on the total esterified fatty acids in both the control and uranium acetate treated rabbits. The fall in total fatty acid level is plotted against the initial level existing prior to the heparin injection. The effect in both the normally fed rabbits and the cholesterol fed rabbits is included in this scatter diagram. Heparin has produced a fall in total esterified fatty acids which is proportional to the initial level of the fraction. There is no apparent reduction in heparin effect on total fatty acids in the uranium acetate treated animals and the points representing this group are distributed fairly well about the same line representing proportional effect as are those in the control. The uranium acetate points tend to be more to the right, because of the higher pre-existing level in these animals, but the proportionate response follows the same pattern (over a wide range of pre-existing levels) as does the control. Thus heparin produces a reduction in the total fatty acids in both control and uranium acetate treated animals, a response which is proportional to the initial level.

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[1] Estimated standard error.

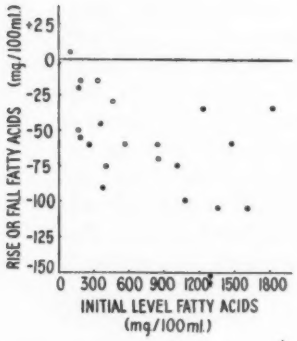

Fig. 5. Reduction in plasma total fatty acid level plotted against initial level in rabbits with varying degrees of lipemia.
○ Control.
● Uranium acetate 10 days before.
Details in text.

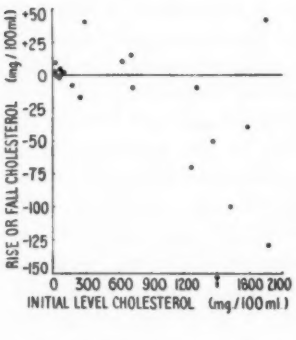

Fig. 6. Reduction in plasma cholesterol level plotted against initial level in rabbits with varying degrees of lipemia.
○ Control.
● Uranium acetate 10 days before.
Details in text.

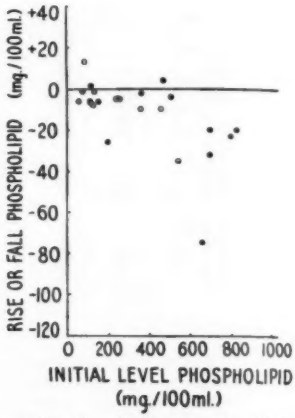

Fig. 7. Reduction in phospholipid level plotted against initial level in rabbits with varying degrees of lipemia.
○ Control.
● Uranium acetate 10 days earlier.
Details in text.

Fig. 6 gives the same data with respect to plasma cholesterol, the fall being plotted against the initial level for both groups and over a range of lipemia. Again the results of Experiment II (normally fed rabbits) and Experiment IV (cholesterol fed rabbits) are included in this scatter diagram. It is apparent that only at higher levels of lipemia does heparin exert a marked effect on the cholesterol level. However, at this point the uranium acetate group and the control group are distributed (as far as can be determined with the relatively small number involved) about the same proportionate response line, i.e. there is no apparent difference in response to heparin between the two groups studied, both showing a similar proportionate reduction in cholesterol level at higher levels of lipemia.

The effect of heparin on the phospholipid level in both groups is plotted in Fig. 7. The results of Experiments II and IV are again combined and the effect of heparin on the phospholipid level at varying levels of lipemia is plotted in the case of both uranium acetate and control animals. Heparin produces a fall in this fraction in both groups, an effect proportionate to the initial level and an effect which does not differ between the two groups studied.

DISCUSSION.

Uranium acetate administration has been shown to inhibit heparin-induced lipemia clearing factor. With a substance as toxic as uranium, it might be

expected that this inhibition of clearing activity would be due to the direct action of the metal in the circulation. The *in vitro* incubation of uranium acetate with active post-heparin plasma did not produce any inhibition, however, and the action of uranium on clearing cannot therefore be explained in such simple terms. Further, the intravenous injection of 5 mg./kg. of uranium acetate 30 minutes before heparin administration did not interfere with the elaboration of normal amounts of clearing factor. Only when the uranium acetate was administered some days prior to the heparin was there any inhibition of clearing activity. This is in contrast to the action of Triton which inhibits clearing factor both *in vitro* and *in vivo* (Brown *et al.*, 1953; Schotz *et al.*, 1957). The action of uranium acetate on lipemia clearing is due therefore to a secondary change brought about by the metal. In view of the predominantly nephrotoxic action of uranium it is probable that the effect shown is due to its action on the kidney. The site of clearing factor formation is unknown, but Jeffries (1954) has shown clearing activity in heparinized blood perfused through abdominal viscera, the lung and the isolated hind limbs. The liver, on the other hand, has been found to destroy clearing factor (Jeffries, 1954; Spitzer and Spitzer, 1956). Le Quire *et al.* (1953) have suggested the reticulo-endothelial system as a possible site, since thorotrast, which blocks this system, was shown to produce a reduction in clearing activity. On the other hand, Seifter and Baeder (1954) have demonstrated inhibition of clearing factor in rats with nephrotoxic nephritis. These latter observations together with the results obtained in this paper suggest that further examination of the kidney as a possible source of clearing factor is warranted. It is plausible to suggest that the inhibition of clearing activity by uranium results from renal necrosis with destruction of clearing factor-producing renal tissue. Recently Rosenman *et al.* (1956, 1957) have reported a causal relationship between hypoproteinemia and hyperlipemia in rats with nephrotoxic nephritis. They have suggested that since albumin is essential for normal clearing (Robinson and French, 1953), a reduction in plasma albumin could produce hyperlipemia by an interference with this function. If uranium (like nephrotoxic nephritis) produced severe hypoalbuminemia, this mechanism could be a possible cause of the hyperlipemia produced by this substance. We have certainly obtained direct evidence of inhibition of clearing activity with a nephrotoxin, but we have produced no evidence that sufficient coincident hypoalbuminemia existed to explain this inhibition. Indeed, it would appear from unpublished data that the inhibition of clearing factor produced by uranium cannot be accounted for by hypoalbuminemia.

The other possibility with regard to the inhibition of clearing by uranium is that clearing factor inhibitors are elaborated possibly from the damaged kidney. Such inhibitors have been described in normal plasma (Hollett and Meng, 1957) and an increase in their amount would explain the results obtained here. Preliminary work has indicated that uranium does produce clearing factor inhibitors, but further work on this aspect together with work on the

rôle of the kidney in the elaboration of clearing factor and possible clearing factor inhibitors is in progress and will be reported later.

It might be noted in passing that in the control cholesterol fed rabbits lipemia clearing factor was elaborated and behaved according to the normal pattern in those animals where the plasma lipemia was relatively low. In the three control animals where plasma turbidity was elevated there was a delay before clearing was manifested. This was possibly due to the phenomenon described by French *et al.* (1955), who found that cholesterol containing lipoproteins in the plasma of cholesterol fed rabbits inhibited clearing by post-heparin plasma. The amount of inhibition described in this present work was not sufficient, however, to delay clearing indefinitely and within 10-20 minutes a continuing fall in optical density became apparent.

Uranium acetate elevated the plasma total fatty acids, cholesterol and phospholipids in normally fed rabbits. In cholesterol fed rabbits, however, only the total fatty acids and phospholipids were increased. These findings in cholesterol fed rabbits confirm the results obtained previously (Day *et al.*, 1957a). The failure to elevate the cholesterol in these animals is probably due to the failing dietary intake in the uranium treated group. This reduction masked the elevating effect of uranium on cholesterol which was shown up in the normally fed rabbits. It might be noted that Heymann and Clark (1945) failed to show lipid changes in rabbits following uranium, but did show changes in other species.

In view of the inhibition of clearing activity by uranium acetate and in view of the marked elevation in serum lipids and in particular total fatty acids caused by it, it would seem reasonable to assert that this uranium hyperlipemia is explicable on the basis of elimination of clearing factor activity. Clearing factor behaves as a lipolytic enzyme whose primary action is hydrolysis of triglycerides (Shore *et al.*, 1953), but secondarily may affect other blood lipids (French *et al.*, 1955; Robinson *et al.*, 1956). The effect of inhibition by uranium, conceding that clearing factor is active in normal fat transport, would therefore be an accumulation of triglycerides and secondarily an accumulation of other plasma lipids.

The foregoing interpretation of the pathogenesis of uranium hyperlipemia is complicated by the findings that while it causes inhibition of clearing factor, there is no difference in the response of serum lipids to heparin in uranium treated animals. In both groups heparin was shown to produce a fall in serum lipids of comparable magnitude. We thus have the anomalous situation of serum lipid changes being recorded in the absence of demonstrable clearing factor. As has been pointed out, the action of clearing factor is considered to be primarily concerned with lipolysis of triglycerides with the consequent reduction in total fatty acids and elevation in free fatty acids. Here the total fatty acid changes do not correlate at all with the clearing factor present. These

observations raise several points regarding the mechanism of heparin action on blood lipids. The action of clearing factor on visible lipemia and on lipolysis of triglycerides may not be a common property and therefore inhibition of one action may occur without alteration in the other. Uranium acetate could conceivably inhibit the effect of clearing factor in reducing the turbidity of an emulsion substrate without interfering with the effect of clearing factor in reducing the plasma lipid level. Another possible explanation is that inhibition of the *in vitro* clearing reaction without inhibition of *in vivo* clearing might occur. The emulsion substrate used is an artificial oil emulsion and the difference detected might, therefore, be an artificial difference. The third possibility is to consider the effect of heparin injection as giving rise to a number of differing activities. Heparin injection produces tributyrinase (Levy and Swank, 1954, 1955) and an acceleration of the migration rate of β-lipoprotein (Comfort, 1953, 1955) as well as the clearing factor it elaborates. Indeed, Levy and Swank (1954, 1955) have suggested a rôle for the heparin produced tributyrinase in lipemia clearing. If uranium acetate inhibited clearing factor, but failed to inhibit other substances elaborated by heparin and active in fat transport the results of this present paper would be explained. It is realised that this suggestion is entirely speculative, particularly as there is a species difference in the association of tributyrinase with clearing factor. Myers *et al.* (1955) have demonstrated an association of these two enzymes following heparin in the dog, rat and man, but not in the rabbit, mouse and guinea pig. The apparent ability to dissociate the heparin effects on lipemia by various inhibiting substances is being further investigated and an attempt made to clarify the mechanism of heparin action on plasma lipemia.

SUMMARY.

Uranium acetate was administered subcutaneously to normally fed rabbits and to cholesterol fed rabbits and 10 days later the effect of heparin on the elaboration of lipemia clearing factor and on the plasma lipids determined. Uranium acetate under these circumstances produced almost complete inhibition of lipemia clearing factor.

In vitro incubation of uranium acetate with active post-heparin plasma produced no diminution in clearing activity. Intravenous administration of uranium acetate 30 minutes before heparin did not interfere with the clearing activity produced.

Uranium acetate elevated the plasma total fatty acids, cholesterol and phospholipid in normally fed rabbits over the 10-day period following the injection. In cholesterol fed rabbits the rate of rise of total fatty acid and phospholipid was accelerated, but the rate of rise of cholesterol was unaltered.

The effect of heparin on the plasma lipids in uranium acetate treated rabbits did not differ from that in the controls. In both groups heparin pro-

duced a reduction in the plasma total fatty acids cholesterol and phospholipid roughly proportional to the initial level of the respective fraction.

The significance of the findings in terms of the pathogenesis of uranium hyperlipemia and with respect to the site of formation and mechanism of action of clearing factor are discussed.

Acknowledgments. We wish to record our indebtedness to Mr. A. E. Bowey of the Pharmacy Department for the preparation of the emulsion substrate used for clearing factor determinations and to Miss Mignon Redway for technical assistance.

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MUTATION TO THIAMINE INDEPENDENCE IN *ESCHERICHIA COLI*

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The acquistion by bacteria of the ability to synthesise an essential nutrient has often been reported and despite extensive studies opinions still differ as to the rôle of mutation or adaptation in the emergence of variants. Recombination studies established the genetic basis of heritable variations in certain strains of *Escherichia coli* (Lederberg and Tatum, 1946), *Serratia marcescens* (Belser and Bunting, 1954) and *Pseudomonas aeruginosa* (Holloway, 1955). Evidence for the genetic origin of such variations in other bacteria, however, depended upon the demonstration of the clonal occurrence of the variants and their spontaneous appearance in a non-selective medium (Cavalli-Sforza and Lederberg, 1953). As early as 1943 Luria and Delbrück described a statistical method which they claimed demonstrated the clonal occurrence and hence mutational origin of phage resistant variants of *E. coli* B. This method was subsequently used by a number of workers (Ryan, Schneider and Ballentine, 1946; Ryan, 1948; Curcho, 1948) in an effort to prove the mutational origin of nutritionally proficient variants. No one, however, was able to show that the change from dependence to independence did in fact occur in a non-selective medium. In 1952 Lederberg and Lederberg devised a technique, replica plating, which besides demonstrating the clonal occurrence of mutants also allowed the indirect selection of the mutants from the parent population. This technique was, therefore, admirably suited to the investigation of the spontaneous appearance of an acquired capacity to synthesise an essential nutrient and in the present study was used to establish the mutational origin of thiamine independent variants of a thiamine requiring strain of *E. coli*.

Materials and Methods.

The organism.

The strain (No. 8196) of *Escherichia coli* used in this study was obtained from the National Collection of Type Cultures, Colindale, London. All work was performed on a line derived from a single cell isolated by the method of Johnstone (1943) and maintained on nutrient agar slopes kept at 4° C. after recovery from dried cultures. The organism grew in glucose-ammonium sulphate medium only in the presence of added thiamine, a concentration of at least 0·003 μg. per ml. being required for optimum growth. Inoculation of large numbers of bacteria on to thiamine free medium, however, consistently gave a few colonies of thiamine independent bacteria indistinguishable from the parent type.

Media and cultural conditions.

The basal agar contained per litre of glass distilled water: $(NH_4)_2SO_4$, 2·0 gm.; Na_2HPO_4, 11·25 gm.; KH_2PO_4, 3·75 gm.; $MgSO_4.7H_2O$, 0·2 gm.; $MnSO_4.4H_2O$, 0·01 gm.; NaCl, 0·01 gm.; $FeSO_4.7H_2O$, 0·0005 gm.; washed agar, 15·0 gm.

Two ml. of a 10 p.c. (w/v) solution of glucose, sterilized by Seitz filtration, was added to each 100 ml. of autoclaved medium.

For liquid cultures Oxoid nutrient broth was used. Nutrient agar was prepared by the addition of 1·5 p.c. (w/v) washed agar to nutrient broth before autoclaving.

All cultures were incubated at 37° C. Liquid cultures were prepared by removing a small amount of surface growth from an overnight nutrient agar slope culture and suspending in sterile saline to give just visible turbidity. One-tenth ml. of this suspension was added to 100 ml. of sterile nutrient broth, giving approximately 10^3 bacteria per ml., and 5 ml. of the inoculated medium were transferred to a 6 in. × ¾ in. sterile Pyrex test tube. The culture was incubated in an inclined position on a horizontal shaker (Kantorowicz, 1951) for 8 hours (the end of the logarithmic phase of growth) after which there were about 5×10^9 viable bacteria per ml.

Preparation of a washed suspension. Five ml. of an 8-hour nutrient broth culture were centrifuged until the supernatant liquid was clear. The packed cells were washed once and resuspended in 5 ml. of sterile saline.

Viable counts. Viable counts were made by the method of Miles and Misra (1938).

Indirect selection of thiamine independent mutants by replica plating.

To initiate an enrichment 1 ml. of a washed suspension containing approximately 5×10^9 viable bacteria per ml. was evenly spread over approximately 50 square cm. (to within 0·5 cm. of the edge) of a 9 mm. diameter nutrient agar plate. After 20 hours' incubation this primary plate (master plate A) was pressed on to a square of sterile velveteen supported by an 8 cm. diameter sponge rubber cylinder. The primary plate was stored at 4° C. until next day and the growth pattern on the velveteen was immediately transferred to two basal agar plates which were examined after 20 hours' incubation for the presence of colonies. A site (9 square mm.) on the master plate, corresponding to a colony on each of the basal agar plates, was removed and the growth suspended in 2 ml. of sterile saline. This suspension, further diluted to give only a few mutant cells per ml., was used to inoculate another nutrient agar plate (master plate B) and the cycle of plating and replicating repeated until well isolated colonies of the thiamine independent bacteria were obtained on the nutrient agar plate.

Estimation of the mutation rate from thiamine dependence to thiamine independence.

Five ml. aliquots from 100 ml. of inoculated nutrient broth (10^3 bacteria per ml.) were transferred to each of eleven 6 in. × ¾ in. sterile Pyrex test tubes and the cultures incubated for 8 hours. A washed suspension was prepared from each culture and the number of mutants in each suspension estimated by spreading 0·5 ml. of the washed suspension evenly over the surface of a well dried thick basal agar plate. After 24 hours' incubation each plate was examined and the number of thiamine independent colonies counted. The number of viable bacteria per ml. of the first and last of the series of eleven replicate washed suspensions was also determined.

RESULTS.

Indirect selection of thiamine independent mutants by replica plating.

Thiamine independent mutants were successfully isolated in pure culture after four cycles of indirect selection on three occasions, clonal congruency being obtained on replica plates throughout each enrichment. Table 1 shows the percentage of mutants at each stage of a typical enrichment.

TABLE 1.

Percentage of mutants to parent type on master plates at each stage of a typical enrichment.

Plate	No. of bacteria plated	No. of mutant colonies	Percentage of mutants to parent type	No. of bacteria removed on agar square (9 sq. mm.)
A	$5 \cdot 0 \times 10^9$	1	0·00000002	$3 \cdot 0 \times 10^8$
B	$3 \cdot 0 \times 10^7$	2	0·000007	$1 \cdot 7 \times 10^8$
C	$1 \cdot 7 \times 10^8$	5	0·003	$8 \cdot 6 \times 10^7$
D	$8 \cdot 6 \times 10^3$	3	0·3	$1 \cdot 5 \times 10^7$
E	$1 \cdot 5 \times 10^3$	150	100	—

The indirect selection of thiamine independent mutants by replica plating was, however, not straightforward. In some attempted isolations a thiamine independent colony appeared on only one of the two basal agar plates, replicated from a single master plate seeded with 5×10^9 parent type bacteria, and the enrichments were abandoned. Consideration of the likely number of cells in mutant microcolonies on primary master plates provided a possible explanation of the failure to produce congruent colonies on basal agar replicas. The number of bacteria on each 9 square mm. of an unincubated master plate A (50 square cm. of the surface being inoculated) was, by proportion, approximately 10^7. Since the number of bacteria removed on 9 square mm. of agar after incubation was 3×10^8 (see Table 1), the number of bacteria on master plate A increased only approximately 30-fold during incubation. Assuming an equal growth rate for mutant and parent type, then each mutant colony prior to replication contained in the order of 30 mutant cells. Lederberg and Lederberg (1952) and Kraft and Braun (1955) estimated that only approximately 3 p.c. of the bacteria were transferred from the master plate to each replica plate. Thus transfer of even a single mutant cell from a colony of 30 mutants on a master plate to congruent sites of both replica plates would be very irregular, as was indeed found in the present study.

Estimation of the mutation rate from thiamine dependence to thiamine independence.

The rate of mutation to the thiamine independent condition was estimated from the distribution of the number of mutants in washed suspensions of sets of eleven replicate cultures (Table 2). The observed median number of mutants per washed suspension, and hence per culture, in Experiment 1 was 10 and m the mean number of mutations as calculated by the method of Lea and Coulson (1949) was 3·9. Substitution in the formula m. log. 2/final population gave the mutation rate as $1 \cdot 2 \times 10^{-10}$ per bacterium per generation. Values of $1 \cdot 5 \times 10^{-10}$ and $2 \cdot 1 \times 10^{-10}$ for the mutation rate were obtained in Experiments 2 and 3 respectively, giving an average mutation rate of $1 \cdot 6 \times 10^{-10}$ per bacterium per generation.

TABLE 2.

Distribution of the number of thiamine independent mutants in replicate cultures.

In each experiment eleven tubes containing 5 ml. aliquots of inoculated nutrient broth (10^8 viable bacteria per ml.) were incubated for 8 hours. A washed suspension was prepared from each culture and the number of thiamine independent mutants in the washed suspension, and hence in the original culture, was estimated by plating an 0·5 ml. amount on to a basal agar plate.

Culture No.	No. of mutants in 0·5 ml. of suspension			No. of viable bacteria per ml. of suspension ($\times 10^9$)		
	Experiment			Experiment		
	1	2	3	1	2	3
1	0	1	2	5·0	3·4	3·9
2	1	1	0	—	—	—
3	2	9	2	—	—	—
4	1	0	1	—	—	—
5	0	1	3	—	—	—
6	1	1	4	—	—	—
7	16	0	0	—	—	—
8	0	2	1	—	—	—
9	1	0	17	—	—	—
10	2	0	1	—	—	—
11	29	1	11	3·6	3·8	4·5

— indicates count not done.

DISCUSSION.

Past attempts to demonstrate the spontaneous appearance of nutritionally proficient variants in populations of exacting bacteria involved some form of direct selection, i.e. growth in the absence of the essential metabolite. The experiments with which Fildes and Whitaker (1948) claimed to show the mutational origin of tryptophan independent variants of the typhoid bacillus were not free from direct selection, since exhaustion of the added tryptophan must have accompanied cessation of growth of the exacting parent and allowed the subsequent growth of existing non-exacting variants.

The introduction of the replica plating technique by Lederberg and Lederberg in 1952 permitted the indirect selection of mutant types from parent populations. Indirect selection differed from direct selection in that the selected line was grown on non-selective medium alone; replicas on selective medium were only used to locate variant colonies on the non-selective medium.

The demonstration of the clonal occurrence of thiamine independent variants and their indirect selection from populations of thiamine dependent *Escherichia coli* achieved in the present study confirmed both the mutational origin of the variants and the suitability of replica plating for the selection of nutritionally proficient variants.

In the present study the low number of cells in mutant microcolonies on primary master plates was suggested as the cause of irregular transfer of mutants to basal agar replicas. This defect, overcome by using a sufficient number of

primary master plates to ensure at least one efficient replication, could, indeed, cause the apparent failure to isolate mutants in a particular study.

SUMMARY.

The replica plating technique was used to show that the change from thiamine dependence to thiamine independence in a strain of *Escherichia coli* was due to a spontaneous mutation. The average mutation rate was estimated to be $1{\cdot}6 \times 10^{-10}$ mutations per bacterium per generation.

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MURRAY VALLEY ENCEPHALITIS ANTIBODIES IN AN ABORIGINAL MISSION IN SOUTH AUSTRALIA

THE APPLICATION OF THE HAEMAGGLUTINATION-INHIBITION TEST

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In a previous paper describing a survey of human sera from the Upper Murray region of South Australia, Warner (1957) reported the finding of a high proportion of antibodies to the Murray Valley encephalitis (MVE) virus in a small sample of aborigines from a Mission situated close to the Murray River. The results suggested that the incidence of MVE antibodies was greater than in the white population of the same area. Since this conclusion, if substantiated, is of considerable interest it was felt important to confirm it. This paper describes the results obtained which uphold the initial observation.

In addition to achieving the primary purpose of this investigation, it was felt that advantage should be taken of the opportunity of applying the haemagglutination-inhibition test for MVE in the field. Some viruses will agglutinate the red blood cells of certain species of animals. Sera containing antibodies against the viruses will inhibit such agglutination and the use of dilutions of serum provides a quantitative test for haemagglutination-inhibition (HI) activity. Sabin and Buescher (1950) first demonstrated the haemagglutinating activity of a virus responsible for one of the arthropod-borne encephalitides. Later the work was extended (Chanock and Sabin, 1953; Casals and Brown, 1954; Clarke and Casals, 1955) to other members of the same group of viruses and methods for HI tests were concurrently developed. Casals and Brown (1954) were also able to divide the arthropod-borne encephalitis viruses into two groups, A and B, according to the temperature and pH at which haemagglutinating activity occurred. Macdonald (1952) demonstrated a haemagglutinin for the Murray Valley encephalitis virus and McLean (1956) showed that it belonged to group B (Casals and Brown, 1954).

Haemagglutination-inhibition tests have not been widely applied to the clinical and epidemiological investigation of the arthropod-borne encephalitides though Southam (1956) describes their application to Japanese B Encephalitis and Chanock, Philpot and Buescher (1955) make brief reference to some experience with them in the same disease. There are no published reports of the use

of the HI test in MVE in the field though Anderson (personal communication) has applied it. Hence it was felt that it would be of some value to apply the test to some sera of which a large proportion were known to contain antibodies to the MVE virus.

MATERIALS AND METHODS.

The population sampled.

The Gerard Mission is a small aboriginal settlement situated in the Upper Murray region of South Australia within a quarter of a mile of the Murray River itself. The total population lies between 70 and and 80 and consists mainly of half-castes, there being only about 10 full-blooded aborigines. From this population 39 specimens of blood were obtained and Table 1 shows the age and sex distribution of the donors of the blood. Most of those from

TABLE 1.

Age and sex distribution of donors of specimens from the Gerard Mission.

Age in years	Males	Females	Totals
5-9	2	2	4
10-14	7	4	11
15-19	3	2	5
20-24	0	1	1
25-29	1	4	5
30-34	3	4	7
35-39	0	1	1
40-44	1	1	2
60	2	0	2
75	1	0	1
TOTALS	20	19	39

whom samples were taken had spent all or nearly all their lives near the Murray River, though a few came from the Nullarbor Plain. One or two may have been, at one time or another, as far north as, or a little further than, the Musgrave Ranges, but it is unlikely that any had been further north than Alice Springs. Probably all the donors of specimens had been in the mission at least since 1951.

Blood specimens.

Specimens of whole blood were removed from the donors on one day (19.6.57) and by the same evening were stored at about $+4°$ C. until the following day when sera were separated and stored frozen at about $-20°$ C.

Complement fixation tests.

The method used was that described by Miles and Howes (1952) using a benzene-extracted mouse brain antigen. All sera were first tested at a dilution of 1:5 and those showing fixation were titrated using twofold serial dilutions of a 1:5 dilution of serum.

Neutralization tests.

All neutralization tests were carried out using the same batch of virus which consisted of a 10 p.c. suspension in 10 p.c. rabbit serum saline of brains from mice previously inoculated intracerebrally with the Adelaide strain of MVE virus.

Volumes of 0·2 ml. of serum were incubated at 37° C. for 2 hours with 0·2 ml. of tenfold serial dilutions (from 10^{-4} to 10^{-8}) of virus suspension. The highest and lowest dilutions were inoculated intracerebrally into groups of 5 or 10 mice, more usually the latter.

The remaining three dilutions were inoculated into groups of 10 mice. With each group of sera being tested a control titration was performed using normal rabbit serum in place of the sera being tested. In the control titrations virus dilutions ranged from 10^{-5} to 10^{-9}. The log LD_{50} of each titration was calculated according to the method of Karber (Finney, 1952). The log neutralization indices (Log NI) were obtained by subtracting the log LD_{50} of the control titration from that of the test. Those sera described as positive in the neutralization test had log NI of greater than 1·6. Those described as equivocal had log NI of 1·0 to 1·6 and those described as negative log NI of less than 1·0.

The Haemagglutination-inhibition (HI) test.

Several variations of the described methods of carrying out the HI test were tried. The methods described are largely those of Clarke and Casals (1955) with modifications where indicated.

Preparation of haemagglutinin.

The method of Chanock and Sabin (1953) was used. Recently weaned mice were inoculated intracerebrally with a 1:1000 dilution of the 9th mouse brain passage of the Melbourne strain of MVE virus. This had been supplied to us as freeze dried mouse-brain material in its 6th passage by Dr. French of the Walter and Eliza Hall Institute in Melbourne. On the 5th day after inoculation, when the mice showed advanced signs of characteristic illness, they were bled by cutting the axillary artery under anaesthesia and allowed to die. The brains were removed under aseptic conditions and were washed free of blood with chilled M/64 KCl-borate buffer at pH 9·0 in normal saline. The washed brains were ground in sand with a chilled pestle and mortar. A 20 p.c. suspension was made with chilled KCl-borate buffer (pH 9·0) and centrifuged at 2,000 r.p.m. for 10 minutes in a refrigerated centrifuge. The supernatant fluid was then centrifuged at 13,000 r.p.m. for 1 hr. in a refrigerated angle centrifuge. The supernatant fluid, whose pH was about 8·0, constituted the stock haemagglutinin and the one batch was used in all the tests described in this paper.

Preparation of red cell suspensions.

One-day-old White Leghorn chickens were decapitated and blood allowed to fall into about four times its volume of Alsever's solution. The suspension was filtered through gauze and was stored at 4° C. up to one week. Before use the cells were washed three times with normal saline and made into a 10 p.c. suspension in normal saline which was kept for up to two days at 4° C.

Immediately before use a 0·25 p.c. suspension of red cells was prepared from a stock 10 p.c. suspension and adjusted to an optical density of 0·50 at 490 mμ in a spectrophotometer. For the titration of haemagglutinin the cell suspension was made up in normal saline whereas for the HI test proper it was made up in the "adjusting buffer" (Clarke and Casals, 1955).

Titration of haemagglutinin.

The procedure used here is that described by Sabin (1956). All manipulations described under this heading were carried out at temperatures between 0° and 4° C.

A series of 0·02 M phosphate buffers covering a range of pH from 6·5 to 7·5 at intervals of 0·1 were prepared. Starting at a dilution of 1:4, serial twofold dilutions of 0·5 ml. quantities of the stock haemagglutinin were carried out in each buffer using 4 × 0·5 inch test tubes. To all tubes 0·5 ml. quantities of standardized cell suspension in saline were added. The tubes were left at 4° C. for 3 hrs., and then examined for haemagglutination according to the method of Chanock and Sabin (1953). The highest dilution of the original brain tissue constituting the haemagglutinin which showed definite agglutination was taken as the titre. This dilution was held to contain one agglutinating unit or dose. The stock haemagglutinin used in the tests described here was titrated after 7 days' storage at 4° C. It was found that the optimum pH was 7·0 and the titre 1:2560.

Preparation of sera.

Sera were removed from storage at − 20° C. and allowed to thaw at room temperature. The serum was diluted 1:4 by adding 0·2 ml. to 0·6 ml. of 0·15 M NaCl. To the diluted serum was added 0·8 ml. of a well-shaken but otherwise untreated 1 p.c. suspension of commercial bentonite in 0·15 M NaCl. The mixture was allowed to stand for 20 minutes, being shaken gently at frequent intervals. Afterwards the mixture was centrifuged at 2,500 r.p.m. for 30 minutes. The supernatant fluid was removed and, to each 1·0 ml., 0·1 ml. of 10 p.c. chick cells in saline was added. The mixture was placed in an icebath for 20 minutes and gently shaken from time to time. The cells were then sedimented in a refrigerated centrifuge and enough KCl-borate buffer at pH 9·0 added to the supernatant fluid to adjust the dilution of the original serum to 1:10. The sera were treated in the afternoon, stored at 4° C. overnight and used for the HI test on the following morning.

The HI test proper.

Treated serum and serial twofold dilutions of it were distributed in volumes of 0·25 ml. in 4 × 0·5 inch test tubes in an icebath which was left at 4° C. for about 4 hrs., and 0·25 ml. of haemagglutinin, diluted in KCl-borate buffer at pH 9·0 to contain 16 units, was added to each tube. Known positive and negative sera were included in each test. Also, one tube of each serum alone was set up to test for the absence of agglutinins for chick red cells.

The tests were left at 1° to 2° C. overnight, and on the following day 0·5 ml. of standardized suspension of chick cells in adjusting buffer (Clarke and Casals, 1955) was added to each tube. The adjusting buffer was so constituted that when the cell suspension was added to the serum-haemagglutinin mixtures at pH 9·0 the resulting pH would be the optimum of 7·0. The tubes were shaken and allowed to settle at 4° C. for 3 hrs. and then examined for agglutination. The highest dilution at which no agglutination occurred was recorded as the inhibitory titre of the serum. In all the tests described here, identical results were obtained with the known positive and negative control sera. None of the sera contained agglutinins for chick red cells.

RESULTS.

Table 2 shows the detailed results obtained with the complement fixation, neutralization and haemagglutination-inhibition tests.

Six sera were anticomplementary and of the 33 remaining 15 fixed complement at a dilution of 1:5 or greater. In fact, 45 p.c. of the sera tested were positive. Of 15 positive in the complement fixation test 13 had positive log neutralization indices (log NI $> 1 \cdot 6$) and two had equivocal log NI (1·0 to 1·6). All sera fixing complement showed haemagglutination-inhibition activity. Neutralization tests were carried out on 38 sera: 26 (68 p.c.) were positive, 8 (21 p.c.) were equivocal and the remainder (11 p.c.) negative (log NI $< 1 \cdot 0$). The haemagglutination-inhibition tests were carried out on 35 sera and 24 (69 p.c.) inhibited agglutination at dilutions of 1:80 to 1:1280. No titres from 1:10 to 1:40 were recorded.

Table 3 compares sera tested by both neutralization and HI tests and shows that there is not exact correspondence between the two tests. Some of those designated as positive and equivocal by the neutralization test were negative by the HI test. Conversely, of those positive by the HI test some showed no

TABLE 2.

Results of serological tests on aborigines from the Gerard Mission.

HI Test Result	Complement Fixation test result				
	Anti-Complementary	<1:5	1:5	1:10	1:20
Not performed	1·3[1]	*	1·7	2·0	
No inhibition	2·0 2·1	1·0 1·6 3·0 >2·2 1·4 2·0 2·7 1·6 0·2			
1:80		2·7 0·1	1·5		
1:160	0·6	2·4 3·4	3·1	2·5	2·9
1:320		1·7 2·5	2·5	1·9	2·3
1:640	1·2	2·2 0·0		2·8 1·2	
1:1280	3·5		2·8	3·1 2·3	3·7
Total Complement Fixation tests	6	18	5	7	3

[1] Log Neutralization Index.
* Neutralization test not performed.

TABLE 3.

Comparison of haemagglutination-inhibition and Neutralization tests on aborigines from Gerard Mission.

		Haemagglutination Inhibition tests			Neutralization tests
		Positive	Negative	Not performed	Total
Neutralization tests	Positive	18	6	2	26
	Equivocal	3	4	1	8
	Negative	3	1	0	4
	Total HI tests	24	11	3	38

evidence of neutralizing activity. There appeared to be no correlations between the titres of the three tests.

The proportion of sera positive by all three tests is 79 p.c. If we include those with equivocal neutralizing antibodies it is 89 p.c.

DISCUSSION.

Considering first the incidence of neutralizing antibodies, in the present investigation we found 26 sera positive and 8 equivocal out of 38 tested. This distribution is not significantly different ($\chi^2_{[2]} = 2{\cdot}88$, $0{\cdot}3 > P > 0{\cdot}2$) from previous findings (Warner, 1957) when, of 14 sera tested, 6 were positive and 5 equivocal. Also, our previous suspicion is confirmed that the incidence of neutralizing antibodies in the aboriginal mission is greater than among the white population in the same area. As we suggested previously (Warner, 1957) this finding can be explained by the facts that the mission is very close to the river and its inhabitants are more exposed to the bites of the river bed mosquitoes than are the white population. Several of the aborigines tested on this occasion were tested previously; of these three had positive results and four equivocal. On this occasion five gave positive results and two equivocal. The results disagreed on four occasions, three that were previously equivocal were now positive and one that was previously positive was now equivocal. However, the differences in log neutralizing indices did not exceed one log in five instances but in the other two, the differences were 1·3 and 2·0 logs respectively. We feel inclined to attribute these findings in part to the natural variability of the tests and in part to the fact that the tests were done at different times. This suggests that one may expect considerable variation in the results of such tests. For this reason, in the present investigation, the numbers of animals used in the neutralization tests were increased over those customarily used so that a more accurate comparison could be made between neutralization indices and HI titres.

The incidence of complement fixing antibodies on the two occasions is not significantly different. Two sera were positive out of eleven in the previous survey (Warner, 1957) and 15 positive out of 33 in the present survey. However, of four that were tested on both occasions, all of which were previously reported as negative, two fixed complement at dilutions of 1:10 and 1:20 respectively in this survey. These represent a greater than twofold and a greater than fourfold difference. Such differences are not very great when it is understood that the tests were carried out at different times by different people. In fact, there is no reason to suppose that antibodies do not fluctuate in potency from time to time.

The interpretation of our results which we have discussed above suggests no reason to believe that there has been any considerable dissemination of the MVE virus in the Upper Murray region of South Australia since the last survey completed before December 10th, 1955. Presumably the survey took place

before the main impact of the encephalitis "season" which occurs in late summer (Anderson, 1954). It is now known that the MVE virus was present in the Murray Valley in the summer of 1955-56. Anderson, Dobrotworsky and Stevenson (1958) demonstrated MVE antibodies in the sera of chickens in the Victorian Murray Valley, but detected only two or three clinical cases of MVE in humans in 1955-56. Anderson at the same time forwarded us some chicken sera from the Upper Murray region of South Australia. Tests on these sera indicated that the MVE virus was present in that region also, but no cases of human disease were reported, and no illnesses resembling encephalitis occurred among the aborigines of the Gerard Mission. However, the question remains whether any of the aborigines in our series were infected in the summer of 1955-56, though in view of the absence of any clinical cases and the conformity of our present results with those of the 1955 survey, it seems likely that most of those with antibodies were not infected during 1955-56. If this is so, it is possible that complement fixing antibodies, as detected with our antigen, may persist for at least six years after infection.

It seems certain that all the aborigines with MVE antibodies, in this series, were infected in the neighbourhood of the Murray Valley. It is known that most of them had lived their lives on the Murray River and that only one or two had been as far north as Alice Springs. Beech, Howes and Miles (1953) have shown that groups of aborigines with high incidences of MVE antibodies occurred only north of Alice Springs. The discovery of an isolated group of people with a high incidence of antibodies to the MVE virus, in a region which the virus is known to visit only occasionally (Anderson, White and McLean, 1954; Miles, 1955; Anderson, personal communication; Warner, 1957) does not support Beech, Howes and Miles (1953) view that a high incidence of antibodies indicates proximity to an enzootic focus of the virus. It suggests rather that the relatively infrequent presence of the virus is sufficient to cause infection in a high proportion of aborigines because they are exposed to wild-life and biting insects. It is true that the incidence reported in some of the groups in the Northern Territory reported by Beech, Howes and Miles (1953) is higher (86 to 89 p.c.) than that found by us (69 p.c.) using approximately comparable neutralizing antibody levels. This may be explained by assuming that when the MVE virus spreads from an enzootic focus in the tropics it does not always extend as far south as the Murray Valley. It follows that a reservoir of the MVE virus may not be in Australia at all but may be further north as suggested by Anderson (personal communication).

The results of titrating the haemagglutinin prepared from MVE virus preparations showed that its optimum activity occurred at pH 7·0 when the test was carried out at 4° C. This places the MVE virus in the B group (Casals and Brown, 1954) of arthropod-borne encephalitides and supports McLean (1956) who reached the same conclusion.

One curious feature of our results is that none of our sera showing HI activity did so at titres ranging from 1:10 to 1:40. They were either entirely negative or reacted at titres of from 1:80 to 1:1280 (Table 2). All sera fixing complement at a dilution of 1:5 or greater and about half of those failing to do so showed HI activity. Thus HI antibodies are in this respect similar to neutralizing antibodies. Also the percentage of sera with HI activity (69 p.c.) is almost the same as that of sera with positive ($>1{\cdot}6$) log neutralization indices (68 p.c.). Beyond these similarities, however, we were unable to show correspondence between neutralizing and HI activity (Table 3). Of 31 sera showing neutralizing activity 10 had no HI activity and of 24 showing HI activity 3 had no neutralizing activity. This is in contrast to Southam (1956) working with Japanese B encephalitis, who found that all sera with HI activity also had neutralizing activity, although all those with neutralizing antibodies did not necessarily contain HI antibodies. While it is true that the majority (21 out of 24) of our sera with HI antibodies showed some neutralizing activity, there appeared to be no correlation between HI titres and log neutralization indices (Table 2). A possible explanation for some of the differences between the HI and neutralization tests may lie in the fact that a Melbourne strain of virus was used for one test and the Adelaide strain for the other. We feel, however, that this is unlikely.

The HI titres observed by us must have persisted for at least one year, since the virus was last detected in the region in the summer of 1955-56. Probably many of the HI titres had persisted since the epidemic of 1951. If, as we think probable, the aborigines were not infected in 1955-56, it seems possible that HI titres of up to 1:1280 persist for as long as six years. This contrasts with the findings of Southam (1956) working with Japanese B encephalitis who states that an HI titre in Japanese children in excess of 1:40 is unusual one year after infection.

It is not possible to assess the value of the HI test in the study of Murray Valley encephalitis since no sera from recent sufferers have been examined. Titre of 1:640 or greater were taken by Southam (1956) to indicate recent Japanese B encephalitis. By comparison, which may not necessarily be valid, it would seem that high titres in the HI test are maintained for long periods after infection with MVE virus, but not with the Japanese B encephalitis virus. In this respect HI antibodies may be similar to neutralizing antibodies. The HI test is delicate and not easy to perform, but is much quicker and requires fewer animals than the neutralization test and may therefore be of greater value. However, as we have shown, there is not exact correspondence between the two tests and obviously further investigation is required.

SUMMARY.

Thirty-nine sera from aborigines from the Upper Murray region of South Australia, whose only exposure to the MVE virus is almost certainly limited to

the Murray Valley itself, were tested for MVE antibodies by neutralization, complement fixation and haemagglutinin-inhibition tests.

At least 68 p.c. of the sera contained antibodies to the MVE virus. This incidence is markedly higher than that of the white population in the same area and for other groups of aborigines south of Alice Springs. This high incidence approaches that reported for some groups of aborigines north of Alice Springs, and from these findings it was concluded that the aborigines in the Upper Murray region were more susceptible to infection with the MVE virus than white people by virtue of their proximity to the river and their greater exposure to biting insects. Also it was concluded that a high incidence of antibodies among groups of aborigines did not necessarily indicate the proximity of a reservoir of the MVE virus.

The results of haemagglutination tests confirmed that the MVE virus belongs to the B group of encephalitis viruses. The results of haemagglutination-inhibition (HI) test on sera did not exactly correspond with those of neutralization tests though most sera with HI activity also showed neutralizing activity. There was no corrrelation between the titres of the two tests. It seems likely that HI antibodies, in common with neutralizing antibodies and a proportion of complement fixing antibodies, persist for a number of years in appreciable titres. However, assessment of the value of HI tests in the study of MVE requires further investigation.

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A COMPARISON OF THE EFFECTS OF BARBITURATES, SALICYLATES, CHLORPROMAZINE, 2,4-DINITROPHENOL AND CYANIDE ON THE SYNTHESIS OF GLUTAMINE BY BRAIN CELLS *IN VITRO*

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A number of agents have recently been shown to uncouple (dissociate) phosphorylation from oxidation in brain mitochondria and homogenates of brain tissue. They include a variety of barbiturates (Brody and Bain, 1954), several salicylates (Penniall, Kalnitsky and Routh, 1956; Brody, 1956) and chlorpromazine (Abood, 1955; Century and Horwitt, 1956).

The question whether the uncoupling effects of these agents actually operate in the intact cell is still in doubt and is of some importance in relation to the hypothesis (Brody, 1955) that uncoupling of phosphorylation may be a mechanism of drug action *in vivo*.

In the present work this question has been approached by determining the effects of selected barbiturates, of salicylates and of chlorpromazine on the synthesis of glutamine by a preparation containing intact brain cells, and comparing them with those of the classical uncoupling agent, 2,4-dinitrophenol (DNP), and of the respiratory poison, hydrogen cyanide. The synthesis of glutamine from glutamic acid and ammonia (Krebs, 1935) is known to depend on a supply of adenosine triphosphate (ATP) (Speck, 1947) and can therefore be expected to be inhibited by agents that uncouple phosphorylation. Since, however, ATP formation may be reduced also by inhibition of the respiration, the effect of the above agents on oxygen consumption was determined at the same time; comparison of effects on glutamine synthesis and on oxygen consumption may then enable one to differentiate between inhibition of glutamine synthesis resulting from uncoupling of phosphorylation and any inhibition secondary to depression of the respiratory rate.

Methods.

General procedure. Guinea-pig cerebral tissue was used throughout. After the animal was killed by a blow on the neck, the cerebral hemispheres were removed, passed through a 40-mesh sieve, and the resultant brei mixed with a spatula to obtain homogeneity. 100 mg. portions of tissue (weighed on a torsion balance) were distributed into Warburg flasks each

containing 3·0 ml. Ringer's phosphate solution in the main compartment and 0·30 ml. 12 p.c. phosphotungstic acid solution in the side-arm.

The composition of the Ringer's solution was as follows (mM): NaCl, 119; KCl, 4·8; $MgSO_4$, 1·2; $CaCl_2$, 1·3; NH_4Cl, 1·0; sodium glutamate, 5; glucose, 5; Na_2HPO_4, 16. The pH was adjusted to 7·3 with HCl. It may be noted that in addition to the usual components this medium contained ammonium chloride and sodium glutamate.

Drugs were dissolved in distilled water; where necessary the solutions were neutralised. They were added to the flasks in a volume of 0·10 ml. before the addition of the tissue.

The tissue was dispersed with a spatula to produce a suspension of fragments of cerebrum containing largely intact cells plus some debris. The flasks were placed in a bath maintained at 37° C. and the oxygen consumption measured after a temperature-equilibration period of 15 min. The gas phase was air. Carbon dioxide was removed by KOH papers, except in experiments with cyanide where mixtures of calcium hydroxide and calcium cyanide solutions were used as described by Robbie (1948). The total time of incubation, including the equilibration period, was 2·25 hours.

The incubation was stopped by tipping the phosphotungstic acid solution into the incubation mixture, after which the contents were transferred, without dilution, to centrifuge tubes. After centrifugation, samples of the supernatant liquid were analysed for ammonia and glutamine.

Duplicate flasks were used in each experiment. Duplicates for oxygen consumption or glutamine (from different flasks) did not differ from each other by more than 10 p.c.; generally they were within 5 p.c.

Glutamine and ammonia estimation. The principle of the method has been described previously (Messer, 1955). Briefly, it consists of the alkaline hydrolysis of the amide-group of glutamine, the alkali being half-saturated potassium hydroxide solution and the operation being carried out in a Conway micro-diffusion dish. The ammonia formed as the result of hydrolysis, plus the free ammonia also present, is termed the "total ammonia". The amount of free ammonia is determined separately and is subtracted from the value for total ammonia to give that for amide ammonia.

The details of the procedure are as follows: Two standard Conway dishes were prepared, each containing 1·00 ml. 1 mM potassium biiodate solution in the inner chamber. 0·75 ml. and 0·50 ml. samples of the supernatant solution obtained after centrifugation of the incubation mixture were placed respectively in the outer chambers of the Conway dishes. After the addition of 0·75 ml. saturated potassium carbonate solution to the first dish and 0·5 ml. saturated potassium hydroxide to the second, the dishes were sealed and the hydrolysis of the amide and diffusion of the ammonia allowed to proceed for 90 min. The biiodate solution in each dish was then titrated with 2 mM barium hydroxide solution from a 0·25 ml. micro-burette. The values for free and total ammonia were given by the results obtained with the first and second dishes respectively.

Other details of the procedure are identical with those described by Conway (1947). Analytical duplicates (for ammonia) were within 2 p.c.

In every experiment the amounts of free and total ammonia present in the brain initially (prior to incubation) were determined and subtracted from the results. These amounts were 0·15-0·25 and 0·70-1·05 μmoles ammonia N per 100 mg. fresh tissue for free ammonia and total ammonia respectively, the amount of initial amide varying between 0·50 and 0·95 μmoles amide N per 100 mg.

Cell-free glutamine synthesis. All the drugs used in the present experiments were tested on a cell-free system containing the glutamine-synthesising enzyme. The enzyme was prepared from sheep's brain as described by Elliott (1951). The composition of the complete system was as follows: "Tris" buffer, pH 7·2, 0·2 M; sodium glutamate, 0·05 M; $MgSO_4$, 0·02 M; NH_4Cl, 0·001 M; adenosine triphosphate (sodium salt), 0·002 M; enzyme solution, 0·05 ml. in a total volume of 3·15 ml. The mixture was incubated at 37° C. for

a period of 40 min., when approximately 75 p.c. of the theoretical amount of amide N had been formed. The rest of the procedure, including the estimation of the amide N, was as described above.

RESULTS.

Normal oxygen consumption and glutamine synthesis. Under the conditions used in the present experiments, the normal consumption of oxygen of the guinea-pig brain varied between 5 and 6 μmoles O_2 per 100 mg. fresh tissue per hour. The rate of consumption was not entirely constant, falling by about 20 p.c. from the first to the second hour of incubation. These results for guinea-pig brain respiring in a medium containing glucose and glutamate are slightly lower than those recorded by other workers (e.g. McIlwain, 1955) with slices.

Fig. 1 illustrates an experiment in which the changes in free and total ammonia N and in amide N have been plotted with respect to time, the amide N being calculated as the difference between total and free ammonia N.

The high initial values for free and total ammonia N are due, of course, to

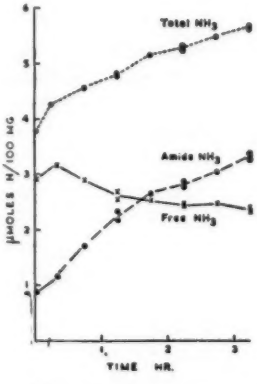

Fig. 1. Time-course of changes in free, total and amide ammonia nitrogen during incubation of guinea-pig brain tissue in Ringer's solution containing glucose, glutamate and ammonium chloride. Amide nitrogen by difference.

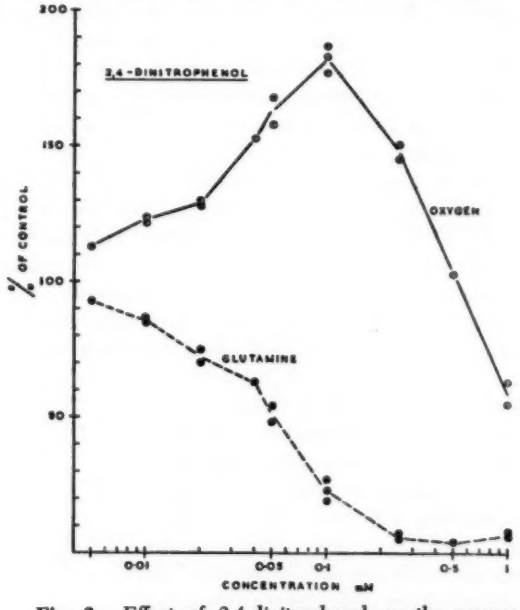

Fig. 2. Effect of 2,4-dinitrophenol on the oxygen consumption and glutamine synthesis of guinea-pig brain. Graph represents the results of six separate experiments. Each point gives the mean result for duplicate flasks.

the presence of added ammonium chloride in the Ringer's solution. It is seen that while the amount of free ammonia N tended to fall (after an initial rise), the total ammonia N showed a marked increase; this increase is a measure of the ammonia formation of the brain. At the same time there was an increase in amide N, representing the synthesis of glutamine. This synthesis varied between 1·8 and 2·6 μmoles amide N per 100 mg. tissue for the usual 2·25 hours of incubation. In general, samples of brain which showed relatively low values for oxygen consumption also gave low results for glutamine synthesis, but this aspect was not further investigated.

Effect of 2,4-dinitrophenol. The presence of 2,4-dinitrophenol in concentrations of 0·005 to 0·1 mM produced marked stimulation of the oxygen consumption and an equally pronounced fall in glutamine synthesis. At concentrations of 0·25 mM and above, initial stimulation of the oxygen uptake was followed by a depression.

These effects are illustrated in Fig. 2, which shows also that the two effects, stimulation of the oxygen consumption and inhibition of glutamine synthesis, appeared to be related in an inverse fashion, up to a concentration of 0·1 mM the degree of respiratory stimulation being roughly equal to the extent of inhibition of glutamine synthesis at all DNP concentrations. At higher concentrations this relationship disappeared but, as already mentioned, at these concentrations the picture was complicated by a supervening inhibition of the oxygen consumption.

It is noteworthy that while maximal inhibition of glutamine synthesis occurred at 0·25 mM DNP, the inhibition of the respiration continued to increase as the DNP concentration was raised still further.

It was of interest to find whether an increase in the magnesium ion concentration of the medium would affect the inhibition of glutamine synthesis produced by DNP. It was found, however, that a fivefold increase in the Mg^{++} concentration (to 6·0 mM) had no effect on the degree of inhibition.

Effect of hydrogen cyanide. Fig. 3 illustrates the effect of graded concentrations of hydrogen cyanide on the oxygen consumption and glutamine synthesis of guinea-pig brain. It is seen that both processes were markedly inhibited, the effect on the former being somewhat greater. The general shape of the glutamine curve, however, closely resembled that of the curve for oxygen consumption, thus indicating a direct relationship between the degree of inhibition of the oxygen uptake and the inhibition of glutamine synthesis.

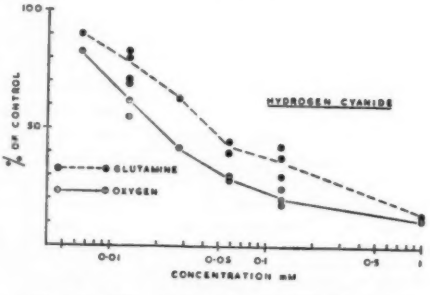

Fig. 3. Effect of hydrogen cyanide on the oxygen consumption and glutamine synthesis of guinea-pig brain. Calcium cyanide-calcium hydroxide mixtures in centre well. Four experiments.

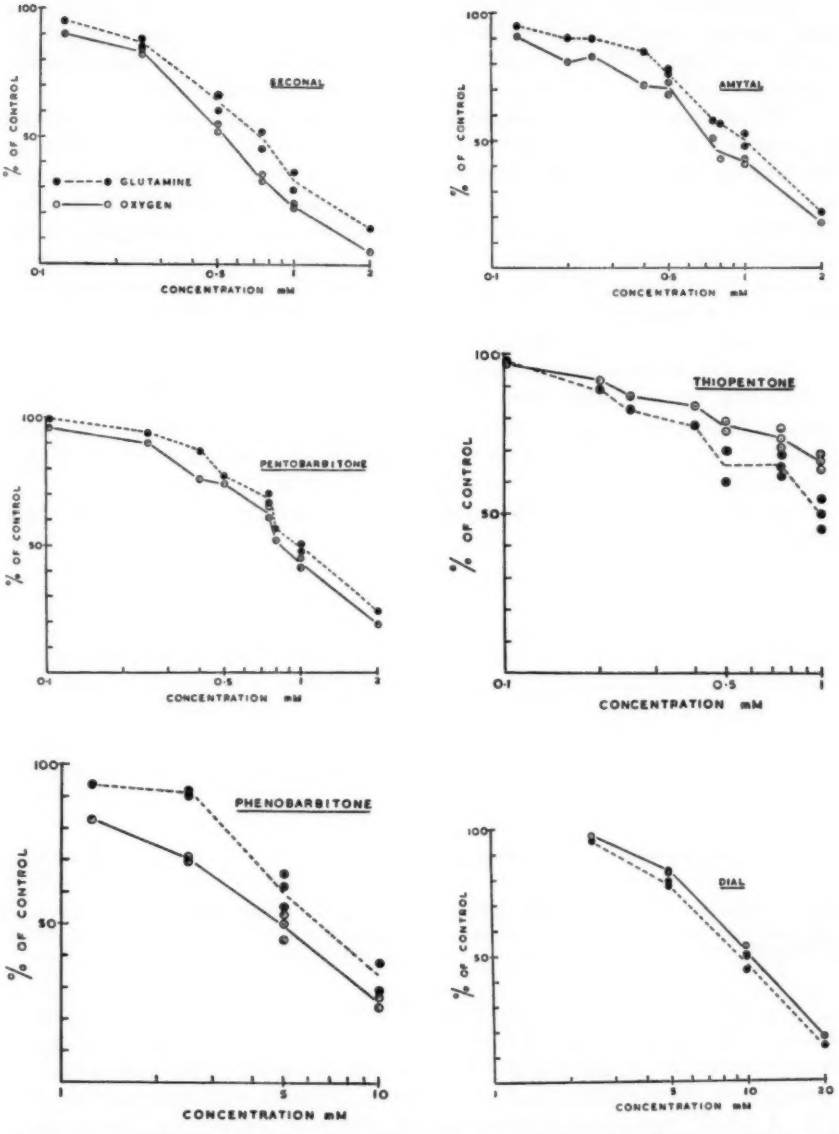

Fig. 4. The effect of barbiturates on the oxygen consumption and glutamine synthesis of guinea-pig brain. Number of experiments: Seconal, amytal, pentobarbitone, 4; thiopentone, 5; phenobarbitone, 3; dial, 2.

Barbiturates. Of the six barbiturates investigated in the present experiments, four—seconal, amytal, pentobarbitone and thiopentone—had been found by Brody and Bain (1954) to exhibit uncoupling activity in brain mitochondria. The other two, phenobarbitone and dial, had had no such effect.

The actions of these barbiturates on the oxygen consumption and synthesis of glutamine are shown in Fig. 4. Three points are worthy of note: 1. None of the barbiturates showed any stimulation of the oxygen consumption over the range of concentrations used. 2. In every case there appeared to be a direct correlation between the degree of depression of the oxygen consumption and the extent of inhibition of glutamine synthesis. 3. There was no apparent difference between the actions of the four "uncoupling" barbiturates on the one hand, and the two "non-uncouplers" on the other, except that the latter acted only at 5 to 10 times higher concentration.

Thus the general pattern shown by the barbiturates resembled the action of hydrogen cyanide rather than that of DNP. Only thiopentone differs somewhat in that, in contrast to cyanide, its effect on the synthesis of glutamine tended to be greater than that on the oxygen consumption, especially at higher concentrations of the drug.

Salicylates. The salicylates investigated were sodium salicylate and sodium acetylsalicylate.

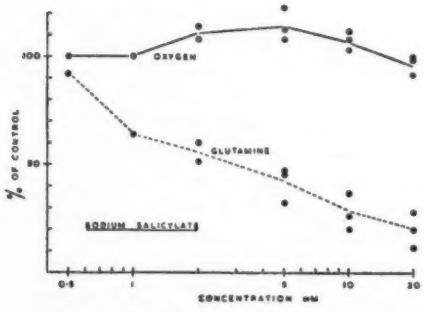

Fig. 5. Effect of sodium salicylate on the oxygen consumption and glutamine synthesis of guinea-pig brain. Five experiments.

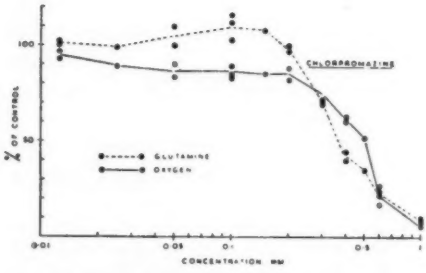

Fig. 6. Effect of chlorpromazine on the oxygen consumption and glutamine synthesis of guinea-pig brain. Seven experiments.

The effect of graded concentrations of sodium salicylate is shown in Fig. 5. It is seen that the drug inhibited the synthesis of glutamine at concentrations at which it either did or did not stimulate the oxygen uptake. The minimum concentration of the salicylate to affect glutamine synthesis was approximately 0·5 mM, at which concentration there was no effect on the oxygen consumption. At concentrations of 20 mM the drug produced an initial stimulation of the oxygen consumption followed by a slight inhibition.

The effect of sodium acetylsalicylate on glutamine synthesis was smaller than that of sodium salicylate (see Table). The former drug, moreover, pro-

TABLE 1.

Comparison of the effects of sodium salicylate and sodium acetylsalicylate on the oxygen consumption and glutamine synthesis of guinea-pig brain.

Salicylate	Concn. (mM)	Percentage of Control	
		Oxygen	Glutamine
Sodium Salicylate	2	114	51
Sodium Acetylsalicylate	2	109	77
Sodium Salicylate	20	92	11
Sodium Acetylsalicylate	20	80	30

duced slightly less stimulation of the oxygen consumption, and greater depression, than did sodium salicylate.

A fivefold increase in the magnesium ion concentration of the medium was found not to affect the action of sodium salicylate on the synthesis of glutamine or oxygen consumption.

Chlorpromazine. The effect of various concentrations of chlorpromazine on the oxygen consumption and glutamine synthesis of the brain cells is shown in Fig. 6.

At concentrations below 0·2 mM the drug produced a small but appreciable inhibition of the oxygen consumption. The extent of this effect showed surprisingly little change over a wide range of concentrations (from 0·025 to 0·2 mM). At the same time, the synthesis of glutamine was either not affected or, in some experiments, tended to increase, particularly at a concentration of 0·1 mM.

At concentrations above 0·2 mM, chlorpromazine produced a sharp and rather sudden inhibition of both oxygen consumption and glutamine synthesis, so that at 1 mM concentration both processes were inhibited by almost 100 p.c. In general, the action of the drug at these concentrations resembled that of the barbiturates, there being a rough correlation between the effect on oxygen consumption and the effect on glutamine synthesis. It is noteworthy, however, that at chlorpromazine concentrations between 0·2 and 0·6 mM the decline in the glutamine curve was markedly more rapid than the decline in the curve for oxygen consumption.

Glutamine synthesis in a cell-free system. In order to find whether any of the drugs used in these experiments exerted a direct effect on the glutamine-synthesising enzyme, they were tested on a cell-free system containing the enzyme, sodium glutamate, ammonium chloride, adenosine triphosphate and magnesium sulphate (see *Methods*). It was found that 2,4-dinitrophenol (1

mM), hydrogen cyanide (1 mM), seconal (1 mM), amytal (1 mM), pentobarbitone (1 mM), thiopentone (1 mM), phenobarbitone (10 mM), dial (20 mM), sodium salicylate (20 mM), and chlorpromazine (1 mM) had no effect on the synthesis of glutamine under these conditions.

Ammonia formation. It was noted that concentrations of drugs which produced inhibition of the oxygen consumption also caused significant depression of the ammonia formation of the brain tissue, as indicated by lower values for total ammonia nitrogen. Since the incubation medium contained a relatively large amount of added free ammonia this effect did not, however, interfere with the synthesis of glutamine.

It was found also that medium concentrations of dinitrophenol (0·02 to 0·1 mM) caused an increase in ammonia formation.

These effects were not further investigated.

DISCUSSION.

The primary purpose of the present work was to obtain information concerning the extent to which the uncoupling of phosphorylation produced by a number of agents in subcellular preparations may affect the metabolism of the intact brain cell.

Theoretical considerations indicated that the synthesis of glutamine might serve as a useful index in this regard. Elliott (1948) and Speck (1949) have shown that glutamine synthesis depends directly on a supply of adenosine triphosphate; in the intact cell, this supply is obtained largely through oxidative phosphorylation with glucose as the substrate. Thus, the synthesis of glutamine might well be expected to be inhibited by agents that uncouple oxidative phosphorylation and which thereby reduce the available energy-supply.

Further, McIlwain (1952) has shown that under the conditions conducive to glutamine synthesis (glutamate as well as glucose in the incubation medium), slices of brain cortex maintain a relatively low level of phosphocreatine. The tissue, thus deprived of a possible reservoir from which it could replenish its energy supply, could therefore be expected to be relatively sensitive to even small changes in the level of available adenosine triphosphate, such as may be induced by uncoupling agents.

This latter consideration appears to have been borne out by the results obtained in the present work with 2,4-dinitrophenol. This drug produced appreciable inhibition of glutamine synthesis at concentrations as low as 0·005 mM (5×10^{-6} M) which were of the same order as those which had been found to be effective in uncoupling phosphorylation in brain homogenates (Case and McIlwain, 1951) or brain mitochondria (Brody and Bain, 1954). The observed inhibition cannot be ascribed to a simple depression of phosphorylation secondary to a respiratory depression since, except at the very highest concentrations, the oxygen consumption was increased rather than diminished. It

was noteworthy, too, that within certain limits of concentration the degree of inhibition of glutamine synthesis induced by the DNP was roughly equal to the degree of stimulation of the oxygen consumption. In other words, the sensitivity of glutamine synthesis to the drug equalled that of the oxygen consumption.

From this one is justified in concluding that the inhibition of glutamine synthesis produced by DNP results from the known uncoupling action of that drug. (Possible interference with the utilization of magnesium ion or direct effects on the enzyme can be excluded on account of the experiments with raised magnesium levels and with the cell-free system, respectively.)

A similar conclusion may be derived from the results obtained with the salicylates. These, too, inhibited the synthesis of glutamine without depressing the oxygen consumption.

The concentrations of salicylate found to be effective in the present experiments were only slightly higher than those found by Brody (1956) to be effective in depressing the P/O in rat brain mitochondria. Brody observed 50 p.c. inhibition of the P/O with 1·5 mM sodium salicylate, while in the present experiments glutamine synthesis was reduced by 50 p.c. at approximately 3 mM salicylate. However, in the experiments of Penniall, Kalnitsky and Routh (1956) the concentrations of salicylate found to inhibit phosphorylation in rat brain mitochondria were somewhat lower than this.

Brody (1956) as well as Penniall *et al.* (1956) found that acetylsalicylate was not as effective in uncoupling phosphorylation as was salicylate. In the present experiments, too, the effect of acetylsalicylate on glutamine synthesis was smaller than that of salicylate. It seems possible that acetylsalicylate as such may have no effect at all, since Lutwak-Mann (1942) has shown that this compound is fairly rapidly hydrolysed by brain.

Brody (1956) has suggested that many of the toxic effects of salicylate in the body may be related to its uncoupling action. The present results would support this hypothesis, since they demonstrate that the uncoupling action hitherto observed only in subcellular preparations also appears to become manifest in the intact brain cell. In this connection it is worth noting that Sproull (1954) has drawn attention to the fact that DNP and salicylates share a number of pharmacological properties.

The results obtained with the barbiturates appear to indicate that the observed inhibition of glutamine synthesis produced by these drugs was merely secondary to the concomitant inhibition of the respiration. In support of this statement, evidence of three kinds may be cited: 1. The action of the barbiturates resembled that of hydrogen cyanide (a drug for which no uncoupling action has, of course, been claimed) in that there appeared to be a direct relation between the depression of the oxygen consumption and the inhibition of glutamine synthesis. 2. There was further resemblance to cyanide in that with one exception (thiopentone) the inhibition of glutamine synthesis was not

appreciably greater than the inhibition of oxygen consumption, contrary to what would be expected if the barbiturates exhibited any appreciable uncoupling activity. 3. There was no essential difference in action between those barbiturates which had been found by Brody and Bain (1954) to uncouple oxidative phosphorylation, and those which had not.

The concentrations of "uncoupling" barbiturates used in the present experiments were between 0·1 and 2 mM. This range is similar to the concentration range (0·1 to 1 mM) in which Brody and Bain (1954) had found barbiturates to uncouple oxidative phosphorylation in brain mitochondria. There is nothing to suggest that the intracellular barbiturate concentration differs essentially from these concentrations. It has recently been shown that thiopentone passes into the dog brain very readily (Mark, Burns, Campomanes, Ngai, Trousof, Papper and Brodie, 1957).

Westfall (1951) has reported that low concentrations of pentobarbitone, amytal and barbitone produced up to 10 p.c. stimulation of the oxygen consumption of brain slices. In the present experiments no such evidence of stimulation could be obtained. However, the concentrations of barbiturates employed by Westfall (1951) were five to ten times lower than those used in the present work and were below the minimum concentration required to uncouple phosphorylation in mitochondria (Brody and Bain, 1954). The experiments of Westfall (1951) are therefore not strictly comparable, nor can they fairly be taken as evidence of uncoupling activity (Brody, 1955).

Webb and Elliott (1951) have shown that barbiturates stimulate aerobic glycolysis of rat brain suspensions; such stimulation is not, however, confined to uncoupling agents (see Elliott, 1955). These authors, too, failed to observe stimulation of the oxygen consumption with barbiturates.

From these considerations it is difficult to avoid the conclusion that the uncoupling activity observed by Brody and Bain (1954) with brain mitochondria has not been demonstrated in experiments with intact brain cells, nor do the present experiments give support to the hypothesis (Brody, 1955) that the hypnotic action of the barbiturates may be based on their ability to uncouple oxidative phosphorylation.

Similar considerations apply to the effects of chlorpromazine observed in the present experiments. This drug, however, presented a slightly more complex picture. At low concentrations (0·1 mM) it tended to increase the synthesis of glutamine, rather than decrease it; at slightly higher concentrations it produced a rapidly developing inhibition of synthesis accompanying, and apparently correlated with, a somewhat less rapidly developing inhibition of the oxygen consumption.

The relative complexity of the action of this drug may possibly be related to the variety of effects chlorpromazine has been found to exert on enzyme systems. Uncoupling of oxidative phosphorylation by chlorpromazine has been observed by Abood (1955) and Bernsohn, Namajuska and Cochrane (1956a)

(but not by Berger, Strecker and Waelsch, 1956) in brain mitochondria and by Decsi (1956) and Century and Horwitt (1956) in brain homogenates. The drug has also been found to inhibit brain flavine enzymes (Yagi, Nagatsu and Ozawa, 1956) as well as brain cytochrome oxidase and adenosine triphosphatase (Bernsohn *et al.*, 1956b). It may well be that in the intact cell any uncoupling effect exerted by chlorpromazine would be counteracted or masked by one or several of these effects on enzymes. Thus, it is possible that the increase in glutamine synthesis caused by low concentrations of the drug in the present experiments may be due to a decreased rate of breakdown of adenosine triphosphate resulting from an inhibition of adenosine triphosphatase. Such inhibition, if it operates in the intact cell, would tend to counteract any uncoupling of phosphorylation. In this connection it may be noted that according to Grenell, Mendelson and McElroy (1955) the brains of rats injected with chlorpromazine showed a much higher content in adenosine triphosphate than those of normal rats.

Thus, although no evidence for an uncoupling effect for chlorpromazine was obtained in the present experiments, such an effect may still operate even in the intact brain cell but would be obscured by the drug's other actions. If that is so, it would be unlikely that uncoupling of phosphorylation by chlorpromazine could be of significance in connection with its known therapeutic or toxic effects.

SUMMARY.

The effects of 2,4-dinitrophenol, hydrogen cyanide, barbiturates, salicylates and chlorpromazine on the synthesis of glutamine and oxygen consumption of guinea-pig brain cells have been investigated.

Dinitrophenol and salicylates (sodium salicylate and sodium acetylsalicylate) inhibited the synthesis of glutamine at concentrations at which they stimulated the oxygen consumption.

Hydrogen cyanide, barbiturates (seconal, amytal, pentobarbitone, thiopentone, phenobarbitone and dial) and chlorpromazine reduced glutamine synthesis but also inhibited the respiration. With these drugs the two effects appeared to be correlated in a direct fashion.

Low concentrations of chlorpromazine tended to increase the synthesis of glutamine.

None of the drugs inhibited glutamine synthesis in a cell-free system.

The results are discussed in relation to the observation made by other workers that barbiturates, salicylates and chlorpromazine uncouple phosphorylation from oxidation in brain mitochondria or homogenates.

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THE INFLUENCE OF ORALLY ADMINISTERED OXY-TETRACYCLINE ON THE RUMEN BACTERIA OF SHEEP

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(*Accepted for publication 12th November, 1957.*)

This work was done because there is a lack of published information about the effects of antibiotics on rumen organisms.

Neumann, Snapp and Gall (1951) reported a disturbance in the bacterial flora and a depression of appetite in steers during prolonged oral dosage with Aureomycin. Perry, Beeson, Hornback and Mohler (1954) also observed depressed appetite with this drug. Chance, Smith, Huffman and Duncan (1953) found, on the other hand, that there was an increase in total bacterial count following the inclusion of Aureomycin in the feed.

Lodge, Miles and Jacobson (1954) found that prolonged Aureomycin administration interferred with cellulose digestion. Huhtanen, Saunders and Gall (1954) observed the same thing with both Aureomycin and Terramycin. Bush, Jacobson and Hartman (1957) noted that Aureomycin levels in the rumen fluid fell sharply within the first three hours.

Horn, Snapp and Gall (1955) recorded an increase in individual facultative anaerobes and a decrease in fibre digestion after giving steers large doses of both Aureomycin and penicillin. Andrews, Gall and Hart (1957) and Andrews, Gall, Hart and Anderson (1957) observed a reduction in fibre digestion in both cobalt-deficient and cobalt-dosed lambs, and the appearance of long segmented rods in cobalt-deficient lambs, following large oral doses of penicillin.

Radisson, Smith and Ward (1956) stated that Terramycin fed to dairy calves caused no marked differences in total and coliform counts, although it significantly decreased the number of enteric streptococci in faeces.

In this paper are recorded the changes in rumen bacteria that were observed, following the oral administration of oxy-tetracycline to sheep.

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MATERIAL AND METHODS.

A mob of sixty Merino-type sheep had been kept as "lawnmowers" for three years on one area, which was overstocked. Twelve were selected; eleven were old, almost or completely toothless, the twelfth sheep was a three-year-old ewe; all were apparently in good health.

The feed consisted of poor quality grass, accessible only in the day time. Water was freely available.

Antibiotic Administered. Oxy-tetracycline (Pfizer's Terramycin, Animal Formula soluble powder) was shaken up with water and given by mouth; each dose contained 1 gm.—equivalent to about 30 mg. per kg. live weight.

The oxy-tetracycline was administered by mouth to 12 sheep, one pair receiving 1 gm. a day on each of three days, one pair on each of two days, one pair on one day; all six were then left untreated for three days before being killed. Of six other sheep one pair received three such daily doses, one pair two daily doses, and one pair one dose; twenty-four hours later they were killed.

Sampling. The rumen content of each sheep was sampled by stomach tube immediately prior to the first dose and, after slaughter, direct from (i) the centre of the rumen, and (ii) near the rumen wall. In every case a 2 oz. sample jar was filled. Sampling was always done prior to feeding.

The samples were immediately taken to the laboratory where the pH was determined.

Bacteriological Methods. Total counts of free-living organisms were made in triplicate, by the direct microscopic method of Moir (1951). Viable counts were made in Gall broth (see Huhtanen, Rogers and Gall, 1952; Huhtanen and Gall, 1953). Smears were made from the original material diluted 1:10, and from each tube in which the broth yielded growth after incubation at 39° C. for two days and again at 10 days, stained and examined microscopically; odour and gas were noted. Sub-cultures were made from representative tubes into deep Gall agar to determine the presence of anaerobic organisms, and smears were made from agar tubes after incubation at 39° C. for 24 to 48 hours.

Dilution Fluid. The dilution fluid was an aqueous solution of (i) 0·75 p.c. $NaHCO_3$, 0·07 p.c. each of (ii) K_2HPO_4 and (iii) KH_2PO_4, dispensed in 90 ml. and 99 ml. lots. Carbon dioxide gas was bubbled through each tube for ½ minute and two drops of the reducing agent added to each immediately prior to use.

Reducing Agent. This was a cysteine HCl solution prepared thus: Add 20 gm. cysteine HCl to 100 ml. of N-NaOH; pH should be *ca.* 7·0. To 4 ml. of this solution add 2 ml. of 7 p.c. $NaHCO_3$. Seal with vaspar. Sterilization: 15 lb. for 15 min.

Media. 10 ml. in each of six tubes containing 1 p.c. each of Difco Bacto peptone, tryptone, beef extract and yeast extract; 0·1 p.c. of K_2HPO_4, KH_2PO_4, and glucose; and two drops of lucerne extract; two drops of the reducing agent; sealed with vaspar seal. Agar medium had the same composition, but with 1·5 p.c. agar added. Sterilization: 15 lb. for 15 min.

Culturing Procedure. The rumen content was strained through sterile muslin, and 10 gm. of the solid material were weighed quickly and added to 90 ml. dilution fluid; then gently shaken; 1 ml. was then transferred to 99 ml. dilution fluid, with a 10 ml. pipette, and from this, 1 ml. to 99 ml. dilution fluid; 1 ml. of this to a test tube containing 4 ml. dilution fluid and 5 ml. Gall broth; from this the culture media were sown in serial dilutions each receiving 1 ml. of the previous tube. The tubes were resealed and incubated at 39° C., read after 2 days, resealed and again incubated for a further 8 days. All samples were sown in duplicate.

RESULTS.

Pre-treatment.

pH of the 12 samples ranged from 6·4 to 7·7, but 75 p.c. of the total fell between 6·6 and 7·2.

Direct smears revealed fairly diverse morphological types typical of those found in the rumen of reasonably well fed sheep; fibres were strongly attacked. (See Fig. 1.)

In the 12 duplicates viable growth terminated with a dilution of 10^7 in two samples, 10^8 in four, 10^9 in 13 samples, 10^{10} in one and at 10^{11} in four samples. However, the smears revealed that dilutions above 10^9 contained morphological types not commonly present in the rumen and perhaps best regarded as contaminants. (Fig. 3.)

The morphological types identified (using Gall's designations) were: lactobacilli, *Str. bovis*, HD, H and SCC from all samples; four samples also contained a few PR. The morphological types were similar in all samples, but the relative numbers varied.

Gas formation was slight, being usually found in tubes with the 10^6 or 10^7 dilution. The faecal odour was pronounced and typical of rumen samples from sheep on grass.

Deep agar tubes sown from the original broth tubes gave typical anaerobic growth with slight gas formation; microscopical examination revealed a typical mixture of several morphological types.

The direct count ranged from 42·2 to 74·4 $\times$ 10^6/mm^3; but 90 p.c. fell between 42 and 60 $\times$ 10^6/mm^3.

Post-treatment.

pH of the 24 samples ranged from 6·0 to 7·5, but 80 p.c. were between 6·4 and 7·2.

The direct smears of samples diluted 1:10 revealed very few morphological types and many grotesque forms; fibres with organisms attached were not found. (Fig. 2.)

In the 12 duplicates viable growth terminated with the 10^6 dilution in one sample, 10^8 in four, 10^9 in four, 10^{10} in seven and 10^{11} in 32 samples.

Morphological types were similar throughout, but only the 10^6 and 10^7 dilutions yielded more than the two morphological types which resembled lactobacilli and *Str. bovis*. These other types did not resemble any organisms usually seen in culture media sown with rumen contents. (Fig. 4.) Only the above two morphological types were identified in the tubes containing 10^9 and above, but many grotesque forms were seen.

Three incidental observations are worth reporting: (1) The direct smears from the last pair of sheep to be treated contained crystals similar to those of oxy-tetracycline, indicating that at least a slight amount was still present in the

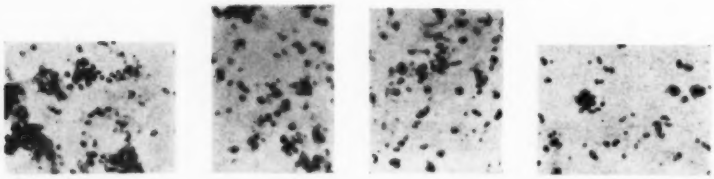

Fig. 1. Gram-stained smear of rumen material diluted 1:10, taken immediately prior to oral treatment of sheep with oxy-tetracycline.

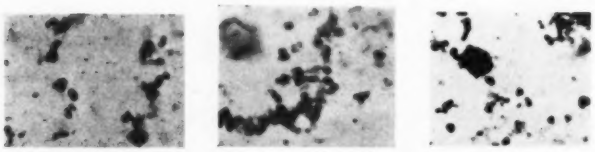

Fig. 2. As above, five days after a single dose of oxy-tetracycline.

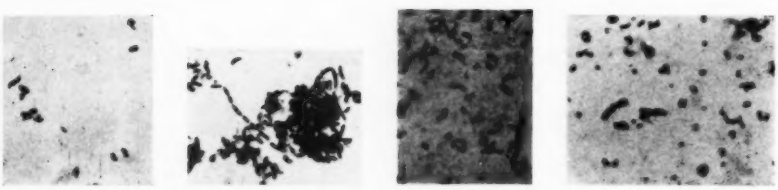

Fig. 3. Crystal violet stained smear of Gall broth, containing rumen material diluted 1:10^6, after incubation at 39° C. for 48 hours, sown prior to treatment with oxy-tetracycline.

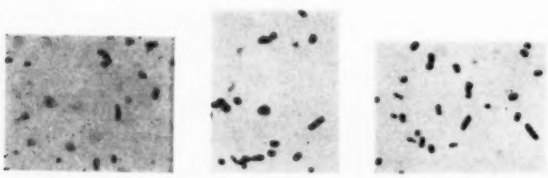

Fig. 4. As for Fig. 3, but rumen sample taken five days after a single dose of oxy-tetracycline.

Magnification for all: *ca.* 1200 diam.

rumen; (2) three sheep showed some damage of the abomasal mucosa indicating that the stomach tube had been pushed into the abomasum; (3) the number of helminths present was negligible.

DISCUSSION.

Following the administration of the antibiotic, there was no obvious change in appetite. On slaughter the rumen of each sheep was well filled with apparently normal contents as was the remainder of the intestinal tract, indicating that the movement of the feed had not been unduly hastened or delayed.

Abnormalities included: (1) a more acid pH of rumen contents; (2) the absence of organisms attached to plant fibres, and (3) the disappearance of typical "rumen bacteria", other than lactobacilli and *Str. bovis*-like organisms. The typical "rumen bacteria" had not returned by the end of the experiment. If pure cultures had been obtained, the true influence of oxy-tetracycline upon specific bacteria might have been determined.

The use of morphological identification is obviously limited, particularly in experiments such as this where "aberrant types" were present in large numbers.

The direct counts were considerably lowered. The error attached to these counts is probably greater than that obtained for counts of rumen content from untreated sheep, as there were apparently many small cells present which were difficult to distinguish from non-cellular matter. The viable counts were considerably increased. Whether this was due to the antibiotic acting as a selective agent is not known. This finding may appear to contradict that of the direct count, and no reasonable explanation for this can be suggested.

It is apparent that there is a lack of published information on the critical dosage levels of antibiotics fed to ruminants. It would seem desirable that the following be determined: (a) the minimum amount of antibiotic which will appreciably change the rumen flora; (b) the time take for rumen flora to revert to normal after treatment ceases.

SUMMARY.

Oxy-tetracycline was given by mouth at different dosage levels to twelve sheep. Their rumen contents were compared before treatment and at autopsy.

The pH of rumen liquid was considerably changed towards the acid range.

The direct counts fell to about ⅓ to ¼ of those found prior to treatment, but viable counts in Gall broth rose considerably.

There was a great decrease in the numbers of organisms attached to fibres.

The typical "rumen organisms" disappeared from the rumen contents with treatment and had not returned by the end of the experiment.

Acknowledgments. Thanks are due to Dr. H. W. Bennetts for advice and friendly criticism, to Mr. G. Munns who made the sheep available, to Mr. R. J. Moir and Mr. E. Walsh for assistance with the photographs, to Mr. S. Gibb for assistance in the field and laboratory, and to the Wool Research Trust Fund for financial assistance.

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TRANSPLANTATION OF THE ADRENAL GLAND OF THE SHEEP TO PROVIDE ACCESS TO ITS BLOOD SUPPLY[1]

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In dealing with any complex phenomenon it is necessary to isolate the different single factors involved for independent study, while keeping the other factors under control. This is the basis of most physiological experimentation. The regulation of sodium balance is a complex phenomenon in which the secretion of the adrenal cortex plays a prominent rôle; but the anatomical location of this organ makes direct access to its blood supply possible only under conditions of anaesthesia and severe surgical trauma. It is known that the gluco-corticoid secretion is under the control of the adenohypophysis, which is itself readily stimulated by relatively minor disturbance in the external and internal environments. Similarly, in experiments designed to evaluate the factors involved in the phenomenon of the relationship between the parotid salivary $Na^+ : K^+$ and Na^+ balance in the sheep (Denton, 1956, 1957) it has been found that one factor is the secretion of the adrenal cortex (Goding and Denton, 1956, 1957; Denton, Goding and McDonald, unpublished) and that factors such as anaesthesia, and possibly emotional disturbance, will interfere with this adrenal component (Denton and McDonald, 1957). Therefore, it was considered necessary to devise a means of isolating the adrenal gland of the sheep in such a way as to have free access to its blood supply without unduly disturbing the animal. This has been achieved by transplanting one adrenal gland to a combined carotid artery-jugular vein loop, with vascular anastomosis, so that by occlusion of the vessels cranial to the transplant, the carotid artery supplies essentially only the adrenal gland, and the jugular vein contains essentially only adrenal venous blood. It has been found that after such transplants, removal of the remaining adrenal gland does not give rise to any evidence of adrenal insufficiency, the relationship between salivary $Na^+ : K^+$ and Na^+ balance is normal, and the renal conservation of Na^+ during Na^+ depletion is normal. Drainage of the venous effluent from such transplants when the sheep is Na^+ depleted causes the salivary $Na^+ : K^+$ ratio to rise, and chromatographic studies of the venous blood of 3 transplanted glands have shown significant adrenal steroid secretion. Histological examination of a transplanted gland showed no abnormality.

[1] This work was aided by grants from the National Health and Medical Research Council, the Wool Industry Fund of the Commonwealth of Australia, and the Rural Credits Fund of the Commonwealth Bank.

Thus it is possible to isolate the adrenal gland in the conscious undisturbed sheep, and examine its function by modifying locally the composition of its arterial blood and assaying the secretory products of its venous blood.

METHODS.

Two Merino wethers, one Merino ewe, and one crossbred wether were used. Another Merino ewe was bilaterally adrenalectomized for control purposes in the subsequent studies of the function of the transplanted adrenal glands.

Chemical methods (Denton, 1956).

Diet.—A standard diet of 0·4 kg. oaten chaff and 0·3-0·4 kg. lucerne chaff was offered daily. This was supplemented by 0·5 kg. fresh lucerne twice weekly when the animals were not on a metabolic balance. A stable preparation of vitamins A and D (Vitamul—Nicholas Pty. Ltd., Melbourne) was mixed with the daily ration to provide 2,500 I.U. and 250 I.U. of each.

Drugs.—Cortisone—"Cortistab", Boots Pure Drug Co. Ltd., Nottingham, England.
ACTH—"Corticotrophin", Commonwealth Serum Laboratories, Melbourne.
Heparin—"Pularin", Evans Medical Supplies Ltd., London, England.
DOCA—"Percorten", Ciba Ltd., Basle, Switzerland.

Surgical procedures.

(1) *Adrenal transplant.*—The operation depends on the fact that the left adrenal gland of the sheep is attached to the left renal vein by a short wide venous trunk 1·0-1·5 cm. from its junction with the inferior vena cava, and one of the arteries supplying the gland arises from the left renal artery near the hilum of the kidney (Fig. 1). The kidney is excised, and segments of the renal artery and vein, which include the junctions with the adrenal vessels, are anastomosed to the carotid artery and jugular vein respectively. In this way the adrenal gland receives its blood from the carotid artery, and drains into the jugular vein. These vessels, together with the transplanted gland, are enclosed in a skin tube, making them readily accessible in the conscious animal. It was found that the Merinos were the most suitable for this operation, because of the large amount of loose skin in the neck.

Anaesthesia is induced by intravenous "Pentothal", and maintained by cyclopropane and oxygen *via* a cuffed endotracheal tube. Normal aseptic precautions are followed. The left adrenal gland is exposed through a loin incision as for adrenalectomy (Goding and Denton, 1957), the exposure being facilitated by the use of a self-retaining retractor, such as the Devine operating frame (Devine, 1940). While one operator prepares the adrenal gland for transplantation, another prepares the vessels in the neck for the anastomosis.

Preparation of the adrenal gland.—The kidney is freed from its fibro-fatty fascia, and drawn up into the wound, exposing the adrenal gland and renal vessels. The tissues surrounding the adrenal gland and renal pedicle are infiltrated with 1 p.c. procaine. The renal vessels are then carefully dissected to separate the entire lengths of renal artery and vein, and the adrenal branch of the renal artery is identified. It can be seen passing round the superior aspect of the renal vein, and down its ventral aspect to the lower pole of the adrenal gland (Fig. 1). By rotating the kidney, this branch can be traced back to its origin near the bifurcation of the renal artery. A small lymph gland near the origin of the adrenal branch assists identification. The dissection of the renal vein is continued down to its junction with the adrenal vein, and before dealing with the interval between the adrenal vein and vena cava, the adrenal gland itself is freed from its adjacent tissues. The superior and middle adrenal arteries (four or five in number) are divided between ligatures. The sympathetic nerve supply is also divided between ligatures. The thoraco-lumbar vein, which sometimes receives a small tributary from the adrenal gland, is ligated. When the adrenal gland is freed so that its only attachments are to the renal artery and vein, the interval between the gland and the inferior vena cava can be cleared. The two limbs of the bifurcated renal artery

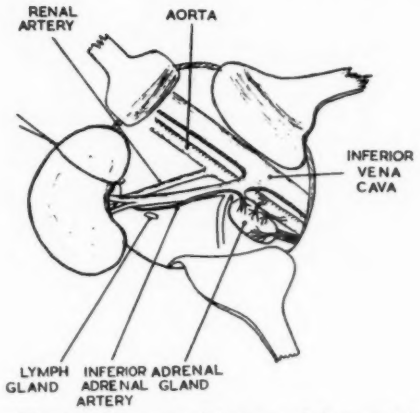

Fig. 1. The essential features of the surgical exposure of the left adrenal gland of the sheep. It is important to identify the inferior adrenal artery before dissecting the renal pedicle extensively. The lymph gland assists identification. The loop of thread around the lower pole of the kidney is a convenient means of retracting this organ, as it can be tied to the operating frame or a towel clip, thus leaving the assistant's hands free.

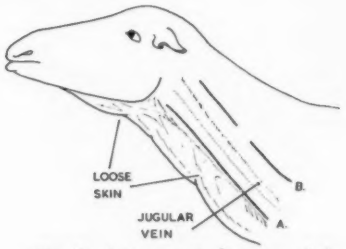

Fig. 2. Approximate location of the skin incisions in the neck, in relation to the course of the jugular vein.

A.—anterior incision, which is made first.

B.—posterior incision, which is interrupted in the middle for approx. 4 cm.

The loose skin in front of the neck of Merino breeds permits easy closure of the skin defect produced by rolling the skin between A and B into a tube.

are then ligated distal to the origin of the adrenal artery, and as close to the kidney as possible. The renal vein is also ligated close to the kidney. A special forceps, similar to a tonsillectomy forceps, but with thinner and longer blades, is applied loosely around the junction of the renal vein and inferior vena cava. When preparation of the neck is completed, the renal artery is clamped near its origin, and immediately this is done, 10 ml. of heparinised saline (100 U. per ml. in 0·85 per cent. NaCl) are slowly injected into the distal part through a fine needle, to wash out the blood in the gland. The introduction of an air bubble must be avoided. The renal vein is then clamped close to the vena cava, and the renal artery and vein are cut free with a scalpel. The kidney is cut away from its vessels distal to the ligatures at the hilum. The adrenal gland, attached by its artery and vein to the renal artery and vein, is immediately transferred to the prepared site in the neck, and the anastomosis is made. Usually, the caval end of the renal vein can be ligated with a silk thread, but if the clamp has been applied to the wall of the vena cava, it will be necessary to oversew the hole with fine arterial silk. The stump of the renal artery is ligated with silk thread.

Preparation of the vessels in the neck.—The carotid artery is exposed by an incision about 15 cm. long extending from 5 cm. below the angle of the jaw towards the thorax, parallel with and 5-7 cm. anterior to the line of the jugular vein (Fig. 2). The carotid sheath is opened, and the vagus nerve is dissected away from the artery. A lymphatic trunk on the posterior aspect of the artery is preserved, as division of this causes troublesome swelling of the face and upper part of the neck in the immediate post-operative period. The carotid artery is freed for about 10 cm. (as much as possible), and the skin with the jugular vein attached is freed from the deeper tissues. The tissue between the jugular vein and the carotid artery and the skin is not disturbed—extensive cleaning of the vein may cause damage to the blood supply of its wall, with subsequent thrombosis. Any large tributaries to the jugular vein are divided between ligatures. A second skin incision is then made 2-3

cm. posterior to the jugular vein, parallel with the first. It is best to interrupt this incision for approx. 4 cm. in the middle, to provide a pedicle for a temporary blood supply to the middle of the loop. The skin between the anterior and posterior incisions is freed from the fascia (but not from the vein) so that it can be rolled into a tube (Fig. 4).

Vascular anastomosis. The renal and carotid arteries are anastomosed end-to-side using a technique similar to that described by Mavor (1956). The middle third of the carotid is isolated by two "bulldog" clips, and a small ellipse is cut out of the wall to correspond with the end of the renal artery, which is cut obliquely. The lumen of the isolated segment is rinsed out with heparinised saline, and the arterial walls are sutured with fine arterial silk. Mavor's method of cutting the vessels and suturing is followed exactly (Fig. 3). The ligatured end of the renal artery is directed cranially. Before completing the arterial suture, the region of the anastomosis is rinsed out with heparinised saline, to remove any trapped air. The upper clip is released first, and any leaking points in the anastomosis are oversewn. When the junction is watertight, both clips are removed, and blood flows from the cut end of the renal vein. The renal vein is anastomosed to the jugular vein in the same way. It is necessary to start the venous suture on the side nearer to the carotid artery.

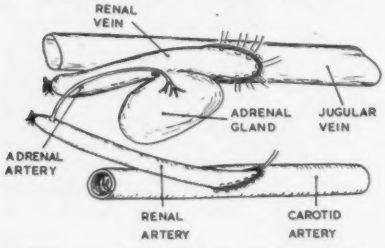

Fig. 3. Diagrammatic representation of the anastomosis between the renal and carotid arteries, and the renal and jugular veins.

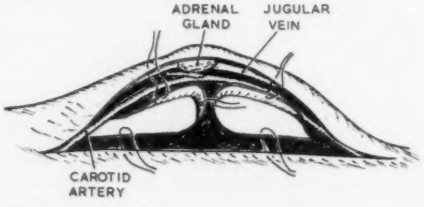

Fig. 4. Method of enfolding the completed transplant with the carotid artery and jugular vein in a tube of skin. The bridge formed by approximating the edges of the skin between the two limbs of the posterior skin incision is divided after healing of the skin tube is complete.

After the anastomosis is completed, the transplant with the carotid artery and jugular vein is enfolded in the freed skin (Fig. 4), and the skin edges are apposed and sutured. In Merino sheep, the resulting skin defect is easily repaired by undercutting and suturing. The edges of the skin intervening between the two parts of the posterior incision are apposed, and sutured together to form a pedicle leading from the skin of the neck to the middle of the tube containing the transplant. This is divided much later, when healing is complete. The end result of the operation (without pedicle) is shown in Fig. 5.

Fig. 5. Transplant No. 1, showing the end result of the transplant operation. The swelling made by the adrenal gland is easily seen (arrow).

Adrenalectomy.—See Goding and Denton (1957).
Parotid fistula.—(Denton, 1957.)

RESULTS.

Study of sheep with adrenal transplants.

The adrenal transplant operation has been performed on four sheep, one of which was sacrificed five weeks later. The other three are alive and in good condition.

Operation histories, pre- and post-operative management.—Each sheep was fasted for twenty-four hours before the operation, and was given $1{\cdot}5 \times 10^6$ I.U. procaine penicillin plus 0·5 gm. streptomycin sulphate daily for four days afterwards.

Transplant No. 1.—A Merino wether, aged 4 years.

3.7.56.—Left adrenal transplant (without pedicle to the middle of the skin tube). There was no special post-operative management. On the day after the operation, a small patch of skin immediately over the transplant was found to be bluish, and this later became necrotic, separating in the second post-operative week. This patch became covered with new epithelium, and healing was complete six weeks after the operation. The swelling in the skin tube, formed by the transplanted gland, did not diminish in size.

10.7.56.—Right adrenalectomy. The sheep was given intramuscular injections of 10 mg. DOCA and 50 mg. cortisone acetate the morning of this operation. It recovered rapidly after the operation, and no supportive hormone treatment was required.

19.7.56.—A right parotid fistula and right carotid artery loop were made. The right cervical sympathetic trunk was divided. The sheep was given 50 gm. $NaHCO_3$ in 1·5 l. of tap water on the second post-operative day, and three times weekly thereafter (Denton, 1957).

5.8.56.—Denervation of the left carotid sinus.

Transplant No. 2.—A Merino ewe, aged 2 years.

24.5.56.—Right adrenalectomy. Intramuscular injections of 10 mg. DOCA and 50 mg. cortisone acetate were given two hours before the operation.

14.6.56.—A right parotid fistula and right carotid artery loop were made. The sheep was given 50 gm. $NaHCO_3$ in 1·5 litres of tap water on the second post-operative day, and twice weekly thereafter.

14.7.56.—Left adrenal transplant (without a pedicle to the middle of the skin tube). The sheep was given 50 gm. $NaHCO_3$ on each of the three days immediately before the operation, and intramuscular injections of 10 mg. DOCA and 50 mg. cortisone acetate late in the day before the operation. Post-operatively, it received intramuscular injections of 10 mg. DOCA on the first and second days, and 40 mg. cortisone on the third day. The supplement of 50 gm. $NaHCO_3$ in 1·5 litres of tap water was given on the second day, and continued every two days. The sheep lost weight and ate very little during the month following the operation, its body weight falling from 32·0 kg. (pre-operative) to 19·2 kg. on the twenty-eighth day. It was given 20 I.U. ACTH and 12·5

mg. cortisone acetate daily for three days from the twenty-second day. During this treatment its appetite improved, it continued to eat well thereafter, and its body weight rose to 23·8 kg. on the thirty-third post-operative day. On the forty-third day, its body weight was 24·7 kg., it was eating normally, and appeared quite strong. It was noted that the swelling formed by the adrenal transplant diminished progressively to about two-thirds its original size by the fourteenth post-operative day, and its size has remained constant since. A small area of skin over the transplant became necrotic and separated in the same way as Transplant No. 1.

Transplant No. 3. A Border Leicester/Merino wether, aged about 5 years.

10.1.57.—Left adrenal transplant (without a pedicle to the middle of the skin tube.) Because the skin of the neck of this sheep was rather tight, difficulty was experienced in mobilising sufficient to form a satisfactory skin tube. Post-operatively, the skin tube became very oedematous, and a large area of skin over the transplant became necrotic. The sheep was given 50 I.U. long-acting ACTH daily from the second to the tenth post-operative day. At the end of this time, a large area of skin, involving almost the whole of the outer aspect of the upper two-thirds of the skin tube, was necrotic and separating. The swelling formed by the transplanted gland was firm, and had not diminished in size. Arterial pulsation could be felt above and below the transplant. The animal was sacrificed on the thirty-fourth post-operative day, and the transplanted gland was carefully examined. The arterial anastomosis was quite satisfactory, and the gland was embedded in the wall of the lower part of the jugular vein, which was patent. The jugular vein above the transplant, in close proximity to the necrotic area, was thrombosed. The cut surface of the transplanted gland appeared normal, and sections were prepared for microscopic examination.

Transplant No. 4.—A Merino wether, aged 4 years.

9.4.57.—Left adrenal transplant. A pedicle to the middle of the skin tube was made, to provide an extra blood supply to this region. The skin immediately over the transplant became bluish for two days after the operation, and then became hyperaemic. During the next three weeks, there was some scaling of the skin in this region, and absence of wool growth, but no other abnormality. Healing has progressed satisfactorily. The swelling formed by the transplant has not diminished in size.

18.4.57.—Right adrenalectomy. The sheep was given intramuscular injections of 10 mg. DOCA and 50 mg. cortisone acetate on the day before the operation, and 5 mg. DOCA and 25 mg. cortisone acetate on the day of the operation. No further hormone injections were given, and the animal is in excellent physical condition.

11.6.57.—Denervation of left carotid sinus. Division of skin bridge to transplant loop.

20.8.57.—Right parotid fistula.

Adrenalectomy No. 5. A Merino ewe, aged 4 years.

22.5.56.—Right adrenalectomy.

7.6.56.—Left adrenalectomy and left nephrectomy. It was originally planned to transplant the left adrenal gland, but the interval between the gland and the inferior vena cava was too small for proper mobilisation, and the renal vein was torn in the attempt. The sheep was given 100 mg. cortisone and 20 mg. DOCA four hours before the operation. After the operation, it was given daily injections of 10 mg. DOCA and 25 mg. cortisone. Many tests have shown that while on the usual dried oats and lucerne diet, evidence of adrenal insufficiency is apparent in this sheep within two days of withdrawal of the hormone injections.

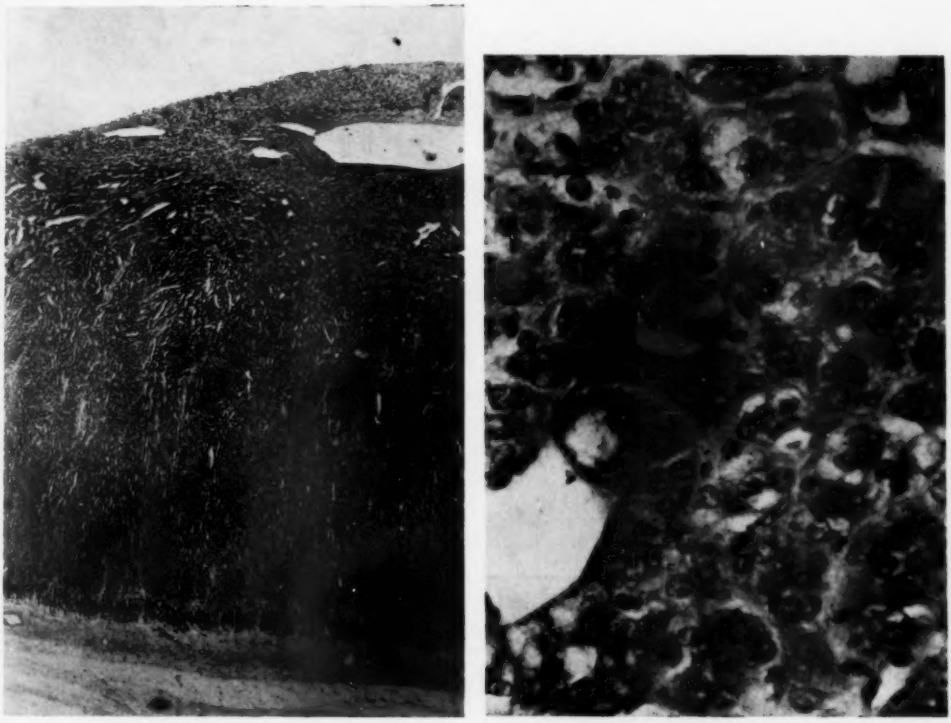

Fig. 6. Transplant No. 3. Microscopic appearance of the transplanted gland. The normal architecture of the gland is preserved, and the differentiation of the several zones appears normal.

(a) General histological arrangement of transplanted gland. (× 20.)

(b) Inner zone of cortex and the medulla. (× 500.) (Celestine blue and eosin.)

Histological appearance of the transplanted adrenal.

The transplanted gland of Transplant No. 3 was fixed in 10 p.c. formol saline and sections were prepared for microscopic examination. The appearance of sections cut across the long axis of the gland, including the adrenal vein, was indistinguishable from the normal (Fig. 6) except for one small area which had become replaced by fibrous tissue. This area was in the upper pole of the gland, which was adjacent to the necrotic region of the loop. The adrenal medulla, as well as the cortex, appeared normal—in keeping with the findings of Levy and Blalock (1939) on the transplanted adrenal of the dog.

Function of the transplanted adrenal.

(1) *Maintenance of life*: Three sheep with transplanted adrenal glands have remained in good condition, their behaviour being indistinguishable from normal

TABLE 1.

The effect of withdrawal of supplementary hormones on the plasma electrolyte concentrations of the sheep with adrenal transplants, compared with a similar episode in a bilaterally adrenalectomized sheep.

Sheep No.	Days after withdrawal of hormones	Plasma				Remarks
		Na^+	K^+	Cl^-	HCO_3^-	
		(m. equiv./l.)				
TR. 1	1	147	5·1	111	—	L. adrenal transplant, 3/7/56.
	2	148	4·7	—	—	R. adrenalectomy, 10/7/56.
	3	148	4·5	113	—	DOCA and cortisone withdrawn, 11/7/56.
	4	147	4·1	113	—	
	6	155	4·7	115	—	
	8	149	4·2	110	—	
TR. 2	(before transplant)	152	4·4	115	28	R. adrenalectomy, 24/5/56. R. parotid fistula and carotid loop, 14/6/56. No $NaHCO_3$ supplement alternate days. L. adrenal transplant, 14/7/56. No $NaHCO_3$ on 3rd day. DOCA and cortisone withdrawn, 16/7/56.
	2	146	5·7	101	28	
	3	139	4·4	119	14	
	8	151	4·6	112	—	
TR. 4	(Before transplant)	150	3·2	112	—	L. adrenal transplant, 9/4/57. 10 mg. DOCA and 50 mg. cortisone, 17/4/57. R. adrenalectomy. 5 mg. DOCA and 25 mg. cortisone, 18/4/57. DOCA and cortisone withdrawn, 19/4/57.
	1	149	3·9	118	—	
	2	149	3·9	118	29	
	5	145	4·8	115	27	
	6	149	4·4	109	27	
	11	141	4·4	114	28	
ADR. 5	(Before withdrawal)	148	4·5	108	30	R. adrenalectomy, 22/5/56. L. adrenalectomy, 7/6/56. Usual hormone supplement = 5 mg. DOCA and 25 mg. cortisone daily. DOCA and cortisone withdrawn 2/7/56.
	1	134	6·6	108	26	
	2	136	7·3	107	21	
	3	129	7·2	106	19	

under laboratory conditions, for six to fifteen months after removal of the second adrenal gland. It is extremely unlikely that there has been significant hypertrophy of accessory adrenal tissue (Goding and Denton, 1957)—see section 4.

(2) *Maintenance of normal plasma electrolyte concentrations*: Withdrawal of the supportive hormone injections from a bilaterally adrenalectomized sheep causes a rapid fall in the plasma Na^+ concentration, and rise in K^+ concentration (Goding and Denton, 1957), and within 3-4 days it is clear that the animal will not survive if the hormone is not replaced. Table 1 shows that the plasma Na^+, K^+, Cl^- and HCO_3^- concentrations of the three sheep with transplants did not alter significantly during eight days from the time the transplanted glands became the only source of adrenal hormones, while gross changes occurred in the plasma of the bilaterally adrenalectomized sheep by the second day after withdrawal of the supplementary hormone injections. For 12 months the plasma electrolyte concentrations of the Transplants Nos. 1 and 2 have remained normal, except during experiments involving disturbance of Na^+ equilibrium, when the changes have been similar to those of sheep with intact adrenals.

(3) *Effect of Na^+ depletion on the composition of the parotid saliva and the urine*: It has been shown elsewhere (Goding and Denton, 1956; Denton, Goding and McDonald, unpublished) that increased secretion of adrenal hormones is a necessary condition for the fall in salivary Na^+:K^+ ratio which normally accompanies Na^+ depletion in the sheep with a permanent parotid fistula (Denton, 1956). The effect of Na^+ depletion on the parotid salivary Na^+:K^+ ratio and urinary Na^+ concentration of the sheep with transplanted adrenals and parotid fistula is the same as in sheep with intact adrenals. In a typical experiment on Transplant No. 1 (Fig. 7), the daily $NaHCO_3$ supplement was

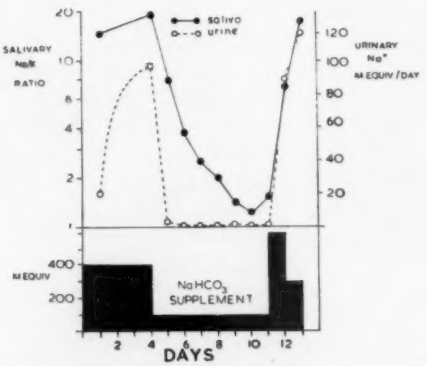

Fig. 7. Transplant No. 1 (with permanent right parotid fistula). The effect on the salivary Na:K ratio and urinary Na^+ excretion of reduction of supplementary $NaHCO_3$ intake, followed by restoration of the $NaHCO_3$ supplement to the maintenance level.

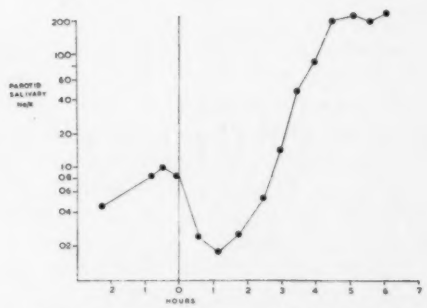

Fig. 8. Transplant No. 1, Na^+ depleted. The effect on the salivary Na:K ratio of administration of 50 gm. $NaHCO_3$ in 1·5 l. tap water into the rumen. The $NaHCO_3$ was administered at hrs. = 0, following control saliva collections over the preceding 3 hours.

reduced from a control level of 35 gm. (417 m.equiv. Na^+) to 8·4 gm. (100 m.equiv. Na^+). At the end of seven days, the Na^+ deficit was 338 m.equiv. The salivary Na^+:K^+ ratio fell from the control level of 19·4 (175:9 m.equiv/l.) to a minimum of 1·2 (93:75 m.equiv/l.) on the sixth day of the seven day period of reduced Na^+ intake. The concentration of Na^+ in the urine fell from 189 to 2 m.equiv/l. by the third day of reduced Na^+ intake, and the total urinary output of Na^+ in the seven day depletion period was 11 m.equiv. compared with 315 m.equiv. in the four day control period. Each of several experiments of this type on Transplant Nos. 1 and 2, and the single experiment on No. 4, has yielded similar results.

Correction of the Na^+ deficit by the intra-ruminal administration of 50 gm. (595 m.equiv.) $NaHCO_3$ caused the salivary Na^+:K^+ ratio to rise to normal after a latent period of two hours (Fig. 8). This rapid increase of salivary Na^+:K^+ ratio following the correction of the Na^+ deficit in Na^+ depleted sheep is due to inhibition of a secretion of the adrenal gland (McDonald and Denton, 1956; Denton and McDonald, 1957). Thus the behaviour of the transplanted adrenal in circumstances of progressive Na^+ depletion and rapid correction of a Na^+ deficit appears to be normal, with reference to renal Na^+ conservation and change in salivary Na^+:K^+.

(4) *Effect of drainage of the transplanted adrenal venous affluent on the composition of the parotid saliva*: By inserting a polythene cannula into the jugular vein on the side of the transplant, so that the tip is in the vicinity of the venous anastomosis, heparinising the animal, and then obstructing the vein above and below the transplant, the venous effluent from the transplanted gland can be drained away, causing a temporary state of adrenal insufficiency. According to the evidence presented by Denton and McDonald (1957) this should cause the salivary Na^+:K^+ ratio of the Na^+-depleted animal to rise. Accordingly, the $NaHCO_3$ supplements of Transplant Nos. 1 and 2 were withdrawn, and when the salivary Na^+:K^+ was lowered, the adrenal venous effluent was drained off. To avoid the consequences of blood loss, compatible blood from a normal non-depleted sheep was infused at a rate corresponding to that of the adrenal venous drainage. After a latent period of 80-100 minutes, the salivary Na^+:K^+ began to rise rapidly, reaching approximately the value found with a high $NaHCO_3$ intake, 200-250 min. after commencement of the adrenal venous drainage (Fig. 9). The adrenal venous effluent was collected into containers immersed in crushed ice, and when the salivary Na^+:K^+ had risen a significant amount, this blood was injected back either into the carotid artery on the side of the parotid fistula (Tr. 2) or into the opposite jugular vein, replacing the donor-blood injection (Tr. 1). The effect was the same in both experiments, in that the salivary Na^+:K^+ ratio fell back to the value preceding the drainage in a time period of 200-250 min. (Fig. 9). The rate of adrenal blood flow in these

experimental conditions was 2·3-3·2 ml./min. This experiment indicates quite clearly that the transplanted glands of both sheep are the only significant source of the adrenal hormones which modify the composition of the parotid saliva, and directly confirms the findings of Denton and McDonald (1957). Another experiment of this type on Tr. 1 caused only a minor rise in salivary Na^+:K^+, but the experimental conditions on this occasion were unsatisfactory, involving unnecessarily severe handling of the animal. The poor parotid response probably indicated that either the activity of the parotid gland was modified by the physical disturbance, or the steroids already present in the circulating blood were not inactivated or excreted. Similar effects have been observed in intact sheep when the Na^+ balance is rapidly changed (Denton and McDonald, 1957). In the two experiments cited above, the polythene cannula was inserted with ease, and the procedure caused only trivial and short-lived disturbance to the animal.

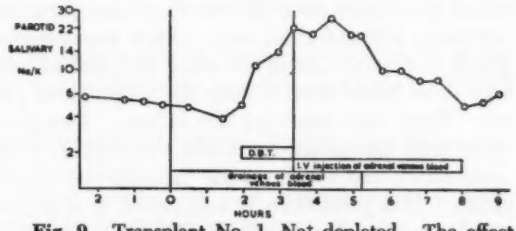

Fig. 9. Transplant No. 1, Na^+ depleted. The effect on the salivary Na:K ratio of draining away the whole of the transplanted adrenal venous effluent through a polythene cannula. D.B.T. = donor blood transfusion, which was given into the right jugular vein to offset the blood loss during the first part of the experiment. The volume of adrenal venous blood collected in the first 3·3 hours was 710 ml.; and the volume of donor blood injected was 300 ml. The volume of adrenal venous blood reinjected into the right jugular vein was 713 ml. Of the remainder, 140 ml. (collected at the beginning of the drainage period) were reserved for chromatographic studies, and 70 ml. were discarded. Total dose of heparin = 10,000 I.U.

(5) *Nature of the steroids secreted by the transplanted glands*: Chromatographic analysis of the adrenal venous effluent of the transplanted glands of Transplants Nos. 1, 2 and 4 in a variety of experimental conditions has been made, and will be the subject of a later communication (McDonald and Reich, unpublished). It has been found that the venous blood of the transplant contains large amounts of compound F and smaller amounts of compound B and aldosterone, the ratio of F:B being approximately 15:1, and the rate of secretion of F varying between 400-600 μg/hr. This quantitative estimate is only approximate, being derived from preliminary work.

COMMENT.

A search of the literature has revealed no comparable procedure for isolating the adrenal gland for study in the conscious undisturbed animal. Levy and Blalock (1939) devised a method for transplanting the adrenal gland of the dog to a subcutaneous site in the neck, using vascular anastomosis. They were concerned only with a means of rapidly removing adrenal tissue without re-

course to a major surgical procedure and their technique of transplantation consequently differed from ours. They transplanted the kidney with the adrenal gland, and anastomosed the renal and carotid arteries end-to-end, relying on the high renal blood flow to keep the anastomosis patent during the period of healing. They then removed the kidney. Dempster (1955) attempted this technique with unsatisfactory results, due mainly to the fact that he made no venous anastomosis and relied on the capsular veins to drain the blood from the whole gland. This procedure is unsuitable if it is desired to manipulate the blood supply to the adrenal gland. First, the artery is relatively inaccessible, being placed deep in the neck and, second, after removal of the kidney, the resulting decrease in blood flow would result in narrowing of the carotid artery to such an extent as to render it unsatisfactory for experimental purposes. An end-to-side anastomosis ensures a good blood flow past the suture line during the period of healing, and does not cause narrowing of the carotid artery. The inclusion of the transplant and vessels in a skin tube permits easy access to the vessels. Inflation of a small pneumatic cuff around the combined loop cranial to the transplant occludes the vessels so that the carotid artery then supplies essentially only the adrenal gland, and the jugular vein contains essentially only adrenal venous blood. A needle can be easily inserted into the carotid artery on the cardiac side of the transplant, and the vein can be compressed between the fingers or by a cuff below the transplant so that the blood withdrawn through a needle or polythene cannula comes only from the adrenal gland, and not from the thorax. A polythene cannula can be inserted into the vein many hours before an experiment, being kept patent by a slow injection of heparinised saline.

It is important to select a suitable animal for transplantation as there must be a sufficient excess of skin in the neck for the construction of a satisfactory skin loop. The Merino breed, with its large dewlap, is the most suitable sheep for this purpose. Our experience with the crossbred sheep, Transplant No. 3, which did not have such a dewlap, illustrates this point, as extensive skin necrosis spoiled an otherwise successful transplant. The problem of skin necrosis in the vicinity of the transplanted gland was also encountered by Levy and Blalock (1939), who attributed it to the release of preformed adrenaline and local ischaemia. The provision of a temporary skin bridge from the neck to the middle of the loop was successful in preventing this complication in Transplant No. 4. Without the extra blood supply provided by this skin bridge it is probable that the skin vessels in this area do not open up quickly enough to prevent permanent damage from the temporary local action of adrenaline released from the gland. Although the necrosis which occurred in Transplant Nos. 1 and 2 was not extensive, and did not vitiate the result of the operation, the relatively simple procedure of providing a temporary skin bridge to the middle of the loop is desirable, as it diminishes this risk of failure.

SUMMARY.

A technique is described for transplantation of the left adrenal gland of the sheep to a prepared site in the neck, so as to render its artery and vein accessible in the conscious animal.

Histological examination of a transplanted adrenal 34 days after the operation showed no significant difference from normal.

Evidence is presented for normal function of such transplants with reference to Na^+ metabolism, and secretion of steroid hormones.

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